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14. Abstract

Project #1

Background: Muscle injuries, especially pulls and strains, are among the most common and most frequently disabling injuries sustained by athletes and soldiers. Although injured muscles heal naturally, the regeneration is very slow and often yields incomplete functional recovery. In injured muscle, regeneration begins shortly after injury, but the healing process is rather inefficient and is hindered by fibrosis—that is, scar tissue formation. More importantly, the scar tissue that often replaces damaged myofibers may contribute to the tendency of strains to recur. We have observed that TGF-β1 plays a central role in skeletal muscle fibrosis and, more importantly, that the use of antifibrosis agents that inactivate this molecule, such as suramin (a Food and Drug Association [FDA]-approved drug that prevents fibrosis due to skin disorders), can reduce muscle fibrosis and consequently improve muscle healing, resulting in nearly complete recovery after laceration or strain injuries.

Objective/Hypothesis/Specific Aims/Study Design: We plan to develop biological approaches based on suramin to efficiently prevent the scarring process by blocking the action of TGF-β1; we will determine the appropriate time at which to administer suramin and the optimal suramin dosage after muscle contusion, a common military injury (**Technical Objective #1**). Because we have observed that suramin can both enhance muscle regeneration and neutralize the fibrotic effect of TGF-β1, we also propose experiments designed to further evaluate the beneficial effects of suramin on muscle regeneration (**Technical Objective #2A**). Finally, we will determine if this effect is mediated through suramin's interaction with muscle growth regulators, particularly its possible down-regulation of myostatin or up-regulation of follistatin (**Technical Objective #2B**).

Supplemental proposal 1 Objectives:
Supplemental Objective 1: To determine the pharmacokinetics of suramin delivery when administered via intramuscular injection in mice for the treatment of skeletal muscle injuries.
Supplemental Objective 2: To evaluate the beneficial effect of decorin, another antifibrosis agent, on muscle regeneration and healing and its mechanism of action.

Supplemental proposal 2 Objectives:

Supplemental Objective 1: To evaluate whether decorin's beneficial effect on muscle healing is mediated through its influence on muscle inflammation. **Hypothesis:** Decorin improves muscle healing by influencing muscle inflammation.

Supplemental Objective 2: To investigate whether decorin's beneficial effect on muscle healing is mediated through MSTN or FLST, two important regulators of muscle growth. **Hypothesis:** Decorin promotes muscle healing by down-regulating MSTN or up-regulating FLST.

Supplemental Objective 3: To evaluate whether angiotensin II receptor blockade after injury represents a potential non-invasive approach to improve muscle regeneration and repair after military-related muscle injury
Hypothesis: Angiotensin II receptor blockade will improve muscle healing after injury.

Relevance: These studies should further our understanding of the muscle healing process, facilitate the identification of new techniques to promote efficient muscle healing, and contribute to the development of innovative therapies for various types of muscle injuries and diseases, such as muscular dystrophies.

Continuation Objectives (4-1-11 to 12-31-11):
Objective 1: Determine the mechanism(s) of action of LOS treatment and refine the roles that follistatin and myostatin play in the regenerative enhancement of injured skeletal muscle with LOS administration.
Objective 2: Determine why our preliminary studies have shown that when LOS is administered at the 3 or 7 days post-injury when compared to administering the drug immediately after injury.

Objective 3: In addition to refining the dosing and timing of LOS administration we are also going to combine the oral administration of LOS with the implantation of muscle derived stem cells and/or along with Platelet Rich Plasma into the contusion injured skeletal muscle of the mice

Project #2

Background: Muscle injuries are very common musculoskeletal problems in traumatology that arise frequently in military training and combat. Injured skeletal muscle undergoes a natural process of regeneration; however, fibrosis—the formation of fibrous scar tissue—hinders this process and precludes the complete recovery of muscle function. Prevention of fibrosis could improve injured skeletal muscle healing; however, it often is not possible to treat muscle injuries before fibrosis occurs.

Objective/Hypothesis: We hypothesize that matrix metalloproteinase type 1 (MMP1), a collagen-digesting enzyme, can improve the microenvironment for muscle regeneration and accelerate healing by digesting existing scar tissue within recovering muscle. Further we hypothesize that MMP-2 can enhance muscle cells migration and regeneration, reorganize muscle structure and accelerate muscle healing after injury.

Specific Aims/Study Design: First, we will culture muscle-derived fibroblasts and myogenic cells in vitro in the presence of MMP1 and assess its effects on fibroblast proliferation and collagen deposition (**Technical Objective #1A**) and myogenic cell migration and differentiation (**Technical Objective #1B**). We then will inject MMP1 directly into normal (noninjured) skeletal muscle to determine the safest and most effective dose of MMP1 in vivo (**Technical Objective #2A**). Next, we will evaluate the ability of MMP1 to digest fibrous scar tissue present within injured skeletal muscle and, by so doing, to enhance the regeneration and functional recovery of injured skeletal muscle (**Technical Objective #2B**). Finally, we will attempt to extend the effective half-life of MMP1 in fibrous scar tissue by genetically engineering myoblasts and muscle-derived stem cells (MDSCs) to express MMP1 (**Technical Objective #3**).

Supplemental Proposal 1 Objectives:

We will evaluate the effects of MMP-2 on myogenic cell migration and differentiation *in vitro* (**Technical Objective #1**). We then will use MMP-2 enhance muscle cells migration and regeneration to improve the functional recovery of injured skeletal muscle *in vivo* (**Technical Objective #2**). Further, we will attempt to assess the relationship between MMP-1 and MMP-2 during muscle healing (**Technical Objective #3**).

Relevance: The study results generated by the proposed project should shed further light on the effects of scar tissue on muscle healing after injury and could facilitate the development of methods by which to eliminate scar tissue and enhance the regeneration of muscle damaged by military- or sports-related injuries or diseases.

Project #3

Our preliminary studies point to a close developmental relationship between vascular endothelial cells and myogenic cells in the adult human muscle. We have, indeed, characterized two novel populations of muscle-derived, non-satellite cells that exhibit dramatic myogenic potential in culture and *in vivo:* genuine vascular endothelial cells and cells co-expressing markers of both myogenic and endothelial cell lineages, which we have named myo-endothelial progenitors. Both cell populations can be purified by flow cytometry as endothelial (CD56- CD 34+ CD144+) and myo-endothelial (CD56+ CD34+ CD144+), and cultured for several weeks without losing their myogenic potential. In our preliminary experiments, the myogenic potential in vivo of myo-endothelial progenitors is dramatically higher than that of endothelial cells, which are themselves much more efficient than regular myogenic cells. We propose experiments to further assess the respective myogenic potentials of human muscle-derived endothelial and myo-endothelial cells, whch will be compared qualitatively and quantitatively following injection into the injured SCID mouse muscle. Besides myogenesis, the development of other cell types, notably endothelial cells and pericytes, upon intramuscular injection will be examined. We hypothesize that upon myo-endothelial cell transplantation, high rates of donor cell survival and/or proliferation will promote skeletal muscle regeneration by generating more donor cells that can

subsequently participate in the regeneration process. We propose a set of experiments to determine the rate at which these different populations of human muscle-derived cells survive and proliferate at different time points after injection, and to evaluate if such differences might affect the regeneration capacity of the cells after transplantation into the skeletal muscle. We will also perform experiments to investigate if these various human muscle-derived cell populations implanted in mice *via* intramuscular injection undergo self-renewal. We will also use a previously described protocol to determine if the donor-derived muscle precursor cells isolated from the skeletal muscle of primary recipient mice can be re-transplanted into the skeletal muscle of secondary recipients and improve skeletal muscle regeneration. To this aim, we will retrovirally modify human-derived myogenic, endothelial and myo-endothelial cells to express GFP. This will enable us to rapidly count the GFP-expressing cells isolated from primary recipients and sort them with high purity for re-transplantation into secondary recipients. Finally, we will also check if cell fusion between donor cells or between donor and host cells plays a significant role in muscle regeneration by these different subsets of human cells.

Supplemental Proposal 1 Objectives:

1- Characterize the potential of pericytes to give rise to a full array of differentiated cells following transplantation into the injured skeletal muscle. We will determine, in a xenochimeric model, whether pericytes can, besides myofibers, regenerate other cell compartments that are fundamental to wound healing and tissue regeneration: blood vessels, connective tissues, nerves.
2- Determine the potential of pericytes to give rise to more primitive stem cells (auto-renewal), and/or to committed muscle progenitor cells (satellite cells) on transplantation into the injured skeletal muscle. Long-term and iterative transplantation experiments will be performed in order to understand whether pericytes can not only regenerate skeletal muscle, but do it permanently. This is a key point to be taken in consideration in the perspective of transplanting these cells into patients.
3- Master the long-term culture of pericytes while maintaining their developmental potential intact. The biology of pericytes *in vitro* will be scrutinized in order to validate culture protocols allowing to obtain large lots of transplantable cells.

Project #4

Background: As progress toward understanding the basic biology of stem cells continues to grow, it is of vital importance that researchers maintain a focus on therapeutic applications of this technology by investigating preclinical models. Members of our laboratory have identified a mouse muscle-derived stem cell (MDSC) population that exhibits a highly enhanced ability to regenerate skeletal muscle in a muscular dystrophy model. Transplantation of this cell population results in significantly more efficient regeneration of skeletal muscle fibers and a significantly larger area of regeneration than does myoblast transplantation, a therapy that has already been tested in human clinical trials in both the United States and Canada. The isolation and transplantation of the human equivalent of these mouse MDSCs likely would improve the outcome of cell therapy for muscular disease and injury, including injuries frequently sustained by military personnel.

Objective/Hypothesis: The objectives of this project are 1) to identify the human stem cell populations that promote the most efficient skeletal muscle regeneration in a preclinical mouse model of muscle regeneration and Duchenne muscular dystrophy (DMD) and 2) to identify the optimal conditions under which to expand cell populations and obtain therapeutically relevant doses.

Study Design: We will screen human muscle-derived cells for a molecular and behavioral profile that correlates with efficient skeletal muscle regeneration in a preclinical model of DMD (Technical Objective #1). We also will identify the cell culturing conditions that best facilitate expansion of the potent populations (to therapeutically useful quantities) while maintaining the cells' phenotype and regeneration efficiency (Technical Objective #2).

Supplemental Proposal 1 Objectives: While ongoing projects are focused on characterizing how to identify and maintain the stem cell phenotype of human MDSC, we are also initiating studies to identify optimal

conditions for the cryostorage of these cells. We will build on the Live cell imaging (LCI) technology as a tool identify the optimal conditions for the processing of the cells as a biologic product for therapeutic applications. LCI provides live viewing of the behavior of a stem cell population in culture and allows us to obtain numerous real-time measurements at the single cell level. The result is a detailed behavioral phenotype of the stem cell population which can be linked to in vivo outcome measures. We will use this tool to follow the changes of cell phenotype in response to temperature changes and fluctuations.

Relevance: This project represents a critical step in cell therapy: Screening potential cell candidates and studying expansion kinetics and limits to generate stem cells for use in cell and gene therapy. This project is unique in that it takes the next step in moving muscle stem cells toward clinical application by investigating how to develop a safe and standardized approach by which to expand stem cell populations. The stem cell screening and expansion techniques that we plan to develop will facilitate the use of cell and gene therapy for myriad musculoskeletal injuries and diseases.

Project # 5

Congenital muscular dystrophy (CMD) is a group of severe forms of muscular dystrophy leading to early death in human patients. The majority of cases are caused by genetic mutations in the major laminin that contains the $\alpha 2$ chain (formerly named merosin) in the muscle basement membrane. The early morbidity/fatality and the lack of effective treatment require urgent search for novel therapeutics. Previously, we utilized mini-agrin, which has been proven to have a therapeutic effect in transgenic MCMD mice, to treat MCMD mice by AAV vector. Our preliminary studies showed that over-expression of mini-agrin protein by AAV vector greatly improved general health and muscle morphology in MCMD mice. However, the treated disease mice still developed gradual paralysis and displayed shorter life span than wild type mice. To further improve the current gene therapy paradigm, with the advanced AAV technology and muscle biology knowledge, we will vigorously test our hypothesis: whether muscle pathogenesis can be improved by inhibition apoptosis or promoting muscle growth. The specific aims are the following:

Aim1: To investigate whether muscle pathogenesis can be improved by delivery of BCL2, an anti-apoptotic gene, by AAV vector in MCMD mice. Mice that lack laminin $\alpha 2$ show severe muscle loss, poor regeneration, and a greatly shortened lifespan. A role for apoptosis in pathology of laminin $\alpha 2$-deficiency has been suggested by histological and in vitro studies, as well as transgenic studies. In this study, we will explore the potential therapeutic effect by delivering AAV-BCL2 vector into MCMD mice.

Aim2: To examine whether therapeutic effect can be obtained by delivery of insulin like growth factor 1 (IGF-1) gene, which can promote muscle growth, by AAV vector in MCMD mice. The MCMD mice show muscle atrophy and enhanced fibrosis as seen in human patients. Genes that promote muscle growth and inhibit fibrosis is theoretically beneficial for congenital muscular dystrophic muscle. Myostatin blockade, one of the strategies to promote muscle growth, has been shown to have a severe side effect of increasing postnatal lethality in MCMD transgenic studies. The reason for the side effect is due to significantly less brown and white fat in the absence of myostatin. In our preliminary studies, we observed that myostatin blockade significantly increased muscle weight, as well as decreased fat tissue in normal mice. However, over-expression of IGF-1 in normal mice only increased muscle weight without loosing fat. Considering less fat will result a severe side effect, we will deliver IGF1 gene to MCMD mice by AAV vector to study whether a therapeutic effect can be achieved in this proposal.

Upon completion, this project will establish complementary therapeutic strategies to combat the severe congenital muscular dystrophy in animal model, setting the base for the development of a clinically efficacious gene therapy strategy.

Project # 6

Background: Elegant studies show that certain cytokines trigger the activation of nuclear factor kappaB (NF-kB) mediated by phosphorylation and degradation of the NF-kB inhibitory protein, IkappaBalpha. Downstream effects of pathological NF-kB activation in skeletal muscle include the inhibition of new muscle formation and the degeneration of existing muscle. *In vitro* studies support the potential that the IkappaBalpha superrepressor (IKBSR), an IkappaBalpha genetically engineered to prevent its phosphorylation, can prevent the activation of

NF-kB in skeletal muscle and could ameliorate or prevent muscle wasting. Our preliminary studies demonstrate the novel determination of inhibition of activation of NF-kB by cFLIP.

Objective/Hypothesis: The proposal will explore the mechanism of muscle wasting in an *in vitro* model and develop novel gene transfer vehicles testing the hypothesis that gene transfer strategies can promote muscle regeneration toward a goal of improving muscle bulk and strength in the setting of injuries or diseases that cause muscle atrophy.

Specific Aims:

Aim 1: To characterize an *in vitro* model of cancer-induced muscle wasting in primary muscle cells and in stable muscle cell lines expressing IKBSR or cFLIP.

Aim 2: To clone, rescue, and purify AAV serotype 1 vectors carrying IKBSR or cFLIP and characterize expression and function *in vitro* in anticipation of future use in an *in vivo* model of muscle wasting.

Study Design: We will characterize an *in vitro* model of muscle wasting studying NF-kB activation, the ubiquitin-proteasome system and caspase activation. We will test whether genetic modifications to muscle cells will confer a benefit by blocking pathways of muscle degeneration. We will develop gene transfer vectors designed to promote muscle regeneration.

Supplemental Proposal 1 Objectives:

Supplemental Objective 1: To test gene transfer approaches in an *in vivo* model of cachexia

A. Test AAV carrying IKBSR for treatment of cancer cachexia

B. Test AAV carrying cFLIP for treatment of cancer cachexia

Supplemental Objective 2: To test peptide transduction approaches of inhibiting NF-κB activation as treatment for muscle cachexia

A. Test peptide transduction in an *in vitro* assay of muscle cachexia

B. Test peptide transduction in an *in vivo* assay of muscle cachexia

Relevance: The ability to promote muscle regeneration in the setting of focal or generalized muscle loss could confer significant clinical benefit in the setting of focal neuropathic or other processes that cause muscle atrophy or chronic illnesses that cause cachexia.

Vector Core:

Background:

The newly established molecular biology laboratory (MBL) will be a Vector Core for the project –Molecular Therapy of Muscle Repair". The goal of this core is to construct vectors such as adeno-associated-, adenoviral- and retoviral vectors for this project. Besides the tissue specific promoter and optimal serotype of vectors in the project #5 and project #6, we will develop a newly modified AAV vector, called the Tet/on and Tet/off AAV vector. This new vector is very useful to ameliorate the acute inflammatory during the early time of muscle injuries. The gene in Tet/on vector will be induced to express the therapeutic gene in the presence of doxycycline, an antibiotic. To avoid the toxicity caused by gene over expression, the vector will be turned off at the time when the muscle is healed.

Objectives:

Objective 1: To continue the production of AAV vectors for the following genes

A: Decorin for project #1

C: Mini-dystrophin gene

D: MPRO (myostatin propeptide) for project # 5

Objective 2: To construct the following genes into AAV vector

A: MMP1 for project #2

B: IKBSR for project #6

C: cFLIP for project #6

Objective 3: To design the new AAV vector containing muscle tissue specific promoter.

Objective 4: To develop the adenoviral and retroviral vectors.

BioReactor Core

The Bioreactor Core serves as a resource for project investigators in need of dynamic cell population analyses. This core uses the Automated Cell CytoWorks™ (ACCW) robotic system to characterize and compare various populations of adult-derived stem cells. The system, in theory, can give the investigator a read out of 20 to 30 different parameters in the form of a –Phenoprint©". We have also planned to set up internet technology to share the information generated in the core facility and thus facilitate further study and development of adult-derived stem cell–based therapies by members of the broader scientific community.

MicroCT Core

Dr. A. Usas operates vivaCT 40 (Scanco Medical) imaging system that enables nondestructive 2-D and 3-D visualization and quantitative analysis of mineralized matrix volume, density and other structural parameters of bone tissue. We are able to perform live animal imaging while they (mice or rats) are put asleep under inhalation anesthesia. VivaCT 40 system allows us to perform multiple scans on the same animal at different time points; therefore we are able to reduce the number of animals required to complete the experiment and cut the cost for animal housing. We can also perform imaging on organs and tissue specimens (calvaria, spine, extremities, muscles, etc.) harvested after animal euthanasia and stored in fixative solution for extended period of time.

We continue to investigate skeletal muscle vasculature using contrast-enhanced micro-CT imaging. One method that has been successfully applied involves transcardiac perfusion and injection of Microfil® silicone rubber compound. After Microfil polymerization that takes 4-6 hours in room temperature blood vessel network in post-mortem tissues of non-surviving animals becomes radiopaque and easily detectable by micro-CT. Another contrast agent for vascular imaging is Fenestra VC, which can be administered repeatedly to animals via single dose administration into tail vein. With Fenestra VC, the delayed uptake by liver cells produces an agent with superior blood pool imaging properties that last for several hours after injection. We are planning pilot experiments to investigate whether Fenestra VC can be used to visualize peripheral vasculature in living mice.

Administrative Core

The Administrative Core of the Stem Cell Research Center (SCRC) is directly responsible for ensuring the proper function and integration of the Research Laboratories (comprising the Core Research Laboratories, Affiliated Laboratories, and Research Core Facilities), the Clinical Trials Unit, and the Educational Programs that constitute the SCRC. The Administrative Core provides administrative services to all SCRC personnel, supports the ongoing activities of the SCRC, and provides a mechanism for regular evaluation of the SCRC. The Administrative Core also is responsible for fulfilling the secretarial, budgetary, and grant application and manuscript preparation needs of SCRC personnel. In addition, this Core also facilitates collaboration between SCRC researchers and scientists working in designated Collaborative Institutes or other, non- affiliated laboratories.

15. SUBJECT TERMS Project #1: Muscle Injuries, suramin, Fibrosis, TGF-beta1, myostatin, follistatin, decorin, Losartan Project #2: Muscular dystrophy, AKT1/Foxo/atrogin1 pathway, glucocorticoids Project #3: Muscle, stem cell, vascular endothelium, tissue regeneration, cell therapy Project #4: muscle regeneration, human myogenic stem cells, expansion, cell aging, population doubling, fibrosis Project #5: Adeno-associated viral vector (AAV),BCL-2, IGF-1, Laminin, myostatin, congenital muscular dystrophy Project #6: Cachexia, muscle, cancer, trauma, adeno-associated virus, gene transfer					
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Table of Contents

Project # 1 Final Report
The use of suramin to improve muscle healing after military-related muscle injuries

(Johnny Huard)

(Project performance dates: 3-1-06 to 12-31-11)

Introduction

Muscle injuries, especially pulls and strains, are among the most common and most frequently disabling injuries sustained by athletes and soldiers. Although injured muscles heal naturally, the regeneration is very slow and often yields incomplete functional recovery. In injured muscle, regeneration begins shortly after injury, but the healing process is rather inefficient and is hindered by fibrosis—that is, scar tissue formation. More importantly, the scar tissue that often replaces damaged myofibers may contribute to the tendency of muscle strains to recur. We have observed that TGF– β1 plays a central role in skeletal muscle fibrosis and, more importantly, that the use of antifibrosis agents that inactivate this molecule, such as suramin (a Food and Drug Association [FDA]-approved drug that prevents fibrosis due to skin disorders), can reduce muscle fibrosis and consequently improve muscle healing, resulting in nearly complete recovery after laceration or strain injuries.

The studies proposed in Technical Objectives 1, 2A and 2B will enable us to determine whether suramin, a drug already approved by the FDA, can improve muscle healing by preventing scar tissue formation after a common military injury (muscle contusion); we also will examine the mechanism(s) by which suramin promotes muscle regeneration. The results of these studies could aid in the development of innovative therapies to promote healing in muscles damaged by different types of injury or disease (e.g., muscular dystrophies), therapeutic approaches that could easily be translated from animal experiments to human clinical applications.

In Supplemental Proposal 1, New Technical Objective 1 we will perform pharmacokinetic studies in mice to evaluate the concentration/ time data of suramin when administered intramuscularly. Our laboratory has shown that intramuscular (IM) injection of suramin after laceration and strain injuries decreases skeletal muscle fibrosis in mice and improves muscle healing, resulting in a near complete recovery of muscle function. In New technical objective 2 we will evaluate the beneficial effect of that decorin, another antifibrotic agent, has on muscle regeneration and healing and its mechanism of action. In preliminary studies we have observed that decorin enhances myogenic differentiation of myoblasts (C2C12 cells) in vitro and that C2C12 cells genetically engineered to express decorin display an enhanced ability to undergo myogenic differentiation in vitro and in vivo. Decorin has also been shown to neutralize the fibrotic effect of TGF-β1 and independently enhance muscle regeneration.

We have observed that direct gene transfer of decorin by injection into uninjured skeletal muscle can decrease the fibrosis and enhance the muscle regeneration observed after subsequent injury of that muscle. In Supplemental Proposal 2, New Technical Objective 1 we will evaluate whether decorin's beneficial effect on muscle healing is mediated through its influence on muscle inflammation. It has been observed that decorin influences the differentiation, survival, and infiltration of macrophages. We, therefore, propose experiments designed to reveal the role of decorin on the inflammatory phase of muscle healing. In Supplemental Proposal 2, New Technical Objective 2 we will also investigate whether decorin's beneficial effect on muscle healing is mediated through MSTN and/or FLST, two important regulators of muscle growth that have been shown to be integral to skeletal muscle regeneration processes. In Supplemental Proposal 2, New Technical Objective 3 we will evaluate yet another antifibrotic agent Losartan. Losartan is an angiotensin II receptor blocker which is cliniocally used for the treatment of hypertensive disorders which was shown to have the unexpected side effect of reducing muscle wasting in these patients. We have preliminary results that demonstrate a beneficial effect form Losartan on skeletal muscle after injury and in this 3rd objective will further study angiotensin II blockade

as a potential non-invasive approach to improve muscle regeneration and repair after military-related muscle injury

Body

1) Original Proposal Objectives

Technical Objective 1: Using an animal model of muscle contusion, a common military injury, we will determine the appropriate dose and time of administration of suramin.

Hypothesis: This is a non-hypothesis driven objective that will rely on empirical data results

Technical Objective 2A: Characterization of the mechanisms by which suramin enhances muscle regeneration will be explored in order to reveal novel ways to promote muscle growth and regeneration.

Hypotheis: Suramin will be shown to effect the key fibrosis and muscle regeneration triggers in skeletal muscle.

Technical Objective 2B: We will investigate suramin's interaction with myostatin and follistatin, 2 important regulators of muscle growth.

Hypothesis: Suramin will down-regulate the expression and up-regulate the expression of follistatin in injured skeletal muscle, thereby improving muscle regeneration and repair.

2) Supplemental Proposal 1 Objectives:

New Technical Objective 1*:* To determine the pharmacokinetics of suramin delivery when administered via intramuscular injection in mice for the treatment of skeletal muscle injuries.

Hypothesis: This is a non-hypothesis driven objective that will rely on empirical data results

New technical objective 2*:* To evaluate the beneficial effect of decorin, another antifibrosis agent, on muscle regeneration and healing and its mechanism of action.

Hypothesis: Decorin will increase muscle regeneration and repair through the enhancement of muscle regeneration and reduction fibrosis.

3) Supplemental Proposal 2 Objectives:

New Technical Objective 1: To evaluate whether decorin's beneficial effect on muscle healing is mediated through its influence on muscle inflammation.

Hypothesis: Decorin improves muscle healing by influencing muscle inflammation.

New Technical Objective 2: To investigate whether decorin's beneficial effect on muscle healing is mediated through MSTN or FLST, two important regulators of muscle growth.

Hypothesis: Decorin promotes muscle healing by down-regulating MSTN or up-regulating FLST.

New Technical Objective 3: To evaluate whether angiotensin II receptor blockade after injury represents a potential non-invasive approach to improve muscle regeneration and repair after military-related muscle injury

Hypothesis: Angiotensin II receptor blockade will improve muscle healing after injury.

4) **Continuation Objectives** (4-1-11 to 12-31-11):
   Objective 1: Determine the mechanism(s) of action of LOS treatment and refine the roles that follistatin and myostatin play in the regenerative enhancement of injured skeletal muscle with LOS administration.
   Objective 2: Determine why our preliminary studies have shown that when LOS is administered at the 3 or 7 days post-injury when compared to administering the drug immediately after injury.
   Objective 3: In addition to refining the dosing and timing of LOS administration we are also going to combine the oral administration of LOS with the implantation of muscle derived stem cells and/or along with Platelet Rich Plasma into the contusion injured skeletal muscle of the mice

Progress to date (3-3-06 to 12-31-11):

a) Results of Original Technical Objective:

It was unknown whether suramin could improve muscle healing after contusion injury, the most commonly encountered muscle injury. Past studies had been performed on mice that had received laceration and strain injuries It also remains unclear whether this enhanced muscle regeneration is a direct effect of suramin. We have performed studies during the first year of funding to examine whether suramin would promote differentiation of myogenic cells *in vitro* and improve injured muscle healing by enhancing regeneration and reducing fibrosis *in vivo*, by using an animal model of muscle contusion.

Muscle-derived stem cell differentiation assay: Muscle-derived stem cells (MDSCs) were isolated from wild type mice (C57BL/6J) via the modified preplate technique. MDSCs (10^4 cells/well) were seeded into 12-well plates and cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum, 10% horse serum, 0.5% chicken embryo extract, and 1% penicillin/streptomycin. After 24 hours, the medium was replaced with differentiation medium (DMEM containing 2% horse serum and 1% penicillin/streptomycin) containing different concentrations of suramin (0, 1, 10, and 100 μL/mL). After another 24 hours, the medium was replaced with differentiation medium. All cells were grown at 37°C in 5% CO_2. Three days after incubation, the fusion index was assessed by counting the number of nuclei in differentiated myotubes as a percentage of the total number of nuclei.

Immunocytochemistry in vitro: Immunocytochemistry was performed on the cells *in vitro* to examine their expression of fast myosin heavy chain (MyHC).

Animal model: The muscle contusion model was developed in normal wild-type mice (7 to10 weeks of age, with an average weight of 24.0g). A 17g stainless steel ball was dropped through an impactor from a height of 100 cm onto the animal's tibialis anterior (TA) muscle. Mice were divided into 4 groups (5 mice/group). Different concentrations of suramin (0, 2.5, 5, and 10 mg in 20 μL of phosphate-buffered saline [PBS]) were injected intramuscularly two weeks after injury. Statistical analysis was performed with ANOVA.

Suramin stimulates MDSC differentiation: Suramin treatment promoted the differentiation of MDSCs *in vitro* in a dose-dependent manner. We observed a significantly higher fusion index in each of the two suramin treatment groups (10 and 100 μg/mL**)** than in the control group (0 μg/mL). Furthermore, 100 μg/mL of suramin treatment enhanced the differentiation significantly more than the other suramin treatments (1 and 10 μg/mL).

Suramin enhances muscle regeneration and decreases fibrosis after contusion injury: We observed a significant increase in the number of regenerating myofibers in all of suramin treated groups (2.5, 5, and 10 mg/20 μL PBS) when compared with the control group (0mg/20μL of PBS).

Moreover, Masson's trichrome staining showed significantly less fibrotic area in all of suramin treated groups than in the control group. Although all three suramin treated groups showed significant improvement in healing by way of the enhancement of muscle regeneration and fibrosis inhibition, there was no significant difference between the three suramin treatment groups.

This data indicate that the suramin-treated MDSC groups have higher fusion indices than the control group *in vitro*. This suggests that suramin can enhance the differentiation of MDSCs, and reveals a portion of the mechanism by which suramin enhances muscle regeneration following injury. This is the first study to show that suramin not only has antiproliferative effects on fibroblasts, it affects the differentiation of MDSCs directly. Our results indicate that suramin can enhance muscle regeneration and prevent fibrosis after a contusion injury, the most common muscle injury **(Refer to Appendices 1, 6 and 7)**. Our future study will investigate the mechanism(s) by which suramin enhances the differentiation of myogenic cells.

We have recently furthered these findings by performing a series of experiments to determine the mechanism (s) behind the beneficial effect of suramin on muscle healing after injury. Our hypothesis was that suramin enhances muscle healing by both stimulating muscle regeneration and preventing fibrosis in contused skeletal muscle. *In vitro*: Myoblasts (C2C12 cells) and muscle-derived stem cells (MDSCs) were cultured with suramin and the potential of suramin to induce their differentiation was evaluated. Furthermore, MDSCs were co-cultured with suramin and myostatin (MSTN) to monitor the capability of suramin to neutralize the effect of MSTN. *In vivo*: Varying concentrations of suramin were injected in the tibialis anterior muscle of mice two weeks after muscle contusion injury. Muscle regeneration and scar tissue formation were evaluated by histological analysis and functional recovery was measured by physiological testing Our results demonstrated that suramin stimulated the differentiation of myoblasts and MDSCs in a dose-dependent manner. Moreover, suramin neutralized the inhibitory effect of MSTN on MDSC differentiation. *In vivo*, suramin treatment significantly promoted muscle regeneration, decreased fibrosis formation, reduced myostatin expression in injured muscle, and increased muscle strength after contusion injury.
In conclusion our results indicate that intramuscular injection of suramin following a contusion injury improved overall skeletal muscle healing. Suramin enhanced myoblast and MDSC differentiation and neutralized MSTN's negative effect on myogenic differentiation *in vitro,* which suggests a possible mechanism for the beneficial effects that this pharmacological agent exhibits *in vivo*. We believe that these findings could contribute to the development of biological treatments to aid in muscle healing after experiencing a muscle injury. These results were recently published in the American Journal of Sports Medicine (**refer to Appendix 7**).

In a final set of experiments, we have examined whether suramin treatment enhances muscle regeneration and reduce fibrosis by down-regulating myostatin expression *in vivo*. The muscle contusion was made on the tibialis anterior (TA) muscle of each mouse. Two weeks after injury, different concentrations of suramin (0 and 2.5 mg) were injected intramuscularly (n=20 mice/ group). At different time points (0.5, 1, 2, 10, and 14 days after injection), mice were sacrificed and cryosections of TA muscle were analyzed histologically. Suramin (2.5 mg) injection demonstrated a significant increase in the number of regenerating myofibers and reduction of fibrotic area when compared with the control group (0 mg). Furthermore, suramin injection effectively inhibited the expression of myostatin in the injured muscle. Our results suggest that suramin improves skeletal muscle healing by enhancing regeneration and reducing fibrosis after contusion injury through a potential decrease in myostatin expression in the injured skeletal muscle. Our findings may contribute to the development of progressive therapies for muscle injury. (**Refer to Appendices 1, 6 and 7)**

b) Results of Supplemental Proposals 1 and 2:

We have shown that decorin, a small leucine-rich proteoglycan, can inhibit TGF-β1 to prevent fibrous scar formation and improve muscle healing after injury. In the decorin-treated muscle, an enhancement of muscle regeneration is observed through histological examination. We have recently determined whether decorin has a direct effect on myogenic cells' differentiation. Our results indicate that myoblasts genetically engineered to express decorin (CD cells) differentiated into myotubes at a significantly higher rate than did control myoblasts (C2C12). This enhanced differentiation led to the up-regulation of myogenic genes (*Myf5, Myf6, MyoD*, and

myogenin) in CD cells *in vitro*. We speculate that the higher rate of differentiation exhibited by the CD cells is due to the up-regulation of follistatin, PGC-1α, p21, and the myogenic genes, and the down-regulation of TGF-β1 and myostatin. Decorin gene transfer *in vivo* promoted skeletal muscle regeneration and accelerated muscle healing after injury. These results suggest that decorin not only prevents fibrosis, but also improves muscle regeneration and repair.

Recent studies have shown that myostatin (MSTN), first identified as a negative regulator of skeletal muscle growth, may also be involved in the formation of fibrosis within skeletal muscle. In a recent study, we further explored the potential fibrotic role of MSTN, as well as its interactions with both transforming growth factor-beta1 (TGF-β1) and decorin. We discovered that MSTN stimulated fibroblast proliferation in vitro, and induced its differentiation into myofibroblasts. We further found that, while TGF-β1 stimulated MSTN expression, MSTN stimulated TGF-β1 secretion in C2C12 myoblasts. Decorin, a small leucine-rich proteoglycan, was found to neutralize the effects of MSTN in both fibroblasts and myoblasts, and up-regulate follistatin (FSTN), an antagonist of MSTN. Moreover, FSTN, an antagonist of MSTN, was up-regulated by decorin. The results of in vivo experiments showed that MSTN-knockout mice developed significantly less fibrosis and displayed better skeletal muscle regeneration when compared to wild-type mice at 2 and 4 weeks following laceration injury. In wild-type mice, we found that MSTN stimulated myofibers to express TGF-β1 in skeletal muscles at early time points following injection. Both TGF-β1 and MSTN were additionally seen to co-localize in myofibers in the early stages of injury. In summary, these findings define a fibrogenic property of MSTN, and indicate a coregulatory relationship between TGF-β1, MSTN, and decorin. Please **refer to Appendices 2, 3 and 4**. The complete recovery of injured skeletal muscle has posed a constant challenge for orthopaedic physician. Once injured, skeletal muscle is able to undergo regeneration from satellite cells; nevertheless, in the serious injured muscle, the formation of fibrosis often impedes effective muscle regeneration and resulted in an incomplete muscle healing. Therefore, to develop biological approaches to improve muscle healing, it is crucial to better understand the mechanisms of the skeletal muscle fibrosis. In the current studies, we found that myostatin (MSTN), a member of TGF-β family, plays a role in the formation of skeletal muscle fibrosis, besides the other putative fibrosis stimulator, TGF-β1. *In vitro*, MSTN directly stimulated the proliferation of fibroblasts and their productions of fibrotic proteins. *In vivo*, after laceration injury, gastrocnemius muscles of MSTN-/- mice showed less fibrosis and better muscle regeneration than wide-type (WT) counterparts. Considering MSTN as a therapeutic target of skeletal muscle healing, we found that inhibitors of MSTN, MSTN propeptide (MPRO) and follistatin, effectively blocked MSTN signaling and improved skeletal muscle healing after injured. We used adeno-associated virus (AAV)-mediated MPRO cDNA to successfully deliver MPRO *in vivo* and improve skeletal muscle healing of normal mice after laceration, and ameliorate dystrophic pathology of *mdx*/SCID mice. Furthermore, our results demonstrated FLST overexpression (FLST/OE) mice exhibited decreased fibrosis and increased muscle regeneration in injured skeletal muscle as compared to wild-type (WT) mice. Moreover, muscle progenitor cells (MPCs) isolated from MSTN-/- and FLST/OE mice significantly regenerated more myofibers than MPCs obtained from WT mice, when transplanted into dystrophic muscles. Collectively, our results suggested that MSTN directly stimulated fibrosis in the injured skeletal muscle; blocking MSTN signaling with MPRO or FLST improved skeletal muscle healing after laceration injury; blocking MSTN signaling in donor MPCs significantly enhanced the success of cell transplantation into dystrophic muscles. Our studies not only uncover some of the mechanisms implicated in skeletal muscle fibrosis and regeneration and help the development of new therapeutic approach for promoting the healing of injured or diseased skeletal muscle, but also renders a new sight of how to obtain robust genetically modified cell populations for cell therapy (**Refer to Appendices 5, 10, 12, and 16**).

Interaction Between Macrophages, TGF-β1, and the COX-2 Pathway During the Inflammatory Phase of Skeletal Muscle Healing After Injury:

An important phase of skeletal muscle healing, largely involves macrophages, TGF-β1, and the COX-2 pathway. To improve our understanding of how these molecules interact during all phases of muscle healing,

we examined their roles in muscle cells *in vitro* and *in vivo*. Initially, we found that depletion of macrophages in muscle tissue led to reduced muscle regeneration. Macrophages may influence healing by inducing the production of TGF-β1 and PGE2 in different muscle cell types. We then found that the addition of TGF-β1 induced PGE2 production in muscle cells, an effect probably mediated by COX-2 enzyme. It was also found that TGF-β1 enhanced macrophage infiltration in wild-type mice after muscle injury. However, this effect was not observed in COX-2_-/-_ mice, suggesting that the effect of TGF-β1 on macrophage infiltration is mediated by the COX-2 pathway. Furthermore, we found that PGE2 can inhibit the expression of TGF-β1. PGE2 and TGF-β1 may be involved in a negative feedback loop balancing the level of fibrosis formation during skeletal muscle healing. In conclusion, our results suggest a complex regulatory mechanism of skeletal muscle healing. Macrophages, TGF-β1, and the COX-2 pathway products may regulate one another's levels and have profound influence on the whole muscle healing process **(Appendix 9).**

Follistatin Improves Skeletal Muscle Healing by Blocking TGF-β-like Signaling Pathway:

Recovery from skeletal muscle injury is often incomplete due to inadequate myofiber regeneration and the formation of fibrosis. Accordingly, injured muscle can benefit significantly from therapies that stimulate muscle regeneration and inhibit fibrosis. To this end, we have focused on doing so by antagonizing a member of the TGF-β superfamily, myostatin. Myostatin is a pharmaceutical target that can be antagonized by follistatin. In vivo, follistatin over-expressing transgenic mice underwent significantly more myofiber regeneration and less fibrosis compared to wild type (WT) mice, as noted after skeletal muscle injury; this is likely partially because follistatin blocks myostatin activity and enhances vacularization. Additionally, the transplantation of muscle progenitor cells, isolated from follistatin over-expressing mice and WT mice, into the skeletal muscle of mdx/SCID mice revealed that the follistatin over-expressing donor cells are significantly superior to WT cells at regenerating skeletal muscle fibers. In vitro, follistatin stimulates myoblasts to express MyoD, Myf5, and myogenin, which are myogenic regulatory factors that promote the myogenic differentiation of myoblasts into myotubes. Furthermore, follistatin induces this enhanced differentiation through the inhibition of myostatin, activin A and transforming growth factor –beta 1, which are negative regulators of myoblast differentiation. This study suggests that follistatin is a promising agent for improving skeletal muscle healing; prior to its pharmacologic application; however, further investigation on follistatin is warranted (**Appendices 5 and 16**).

AAV-Mediated Myostatin Propeptide Gene Transfer Improved the Healing of Laceration Injured Skeletal Muscle and Muscle Progenitor Cell Transplantation Efficiency:

The development of approaches to improve muscle healing after injuries has focused largely on inhibiting fibrosis and promoting myogenesis. Since the inhibition of myostatin (MSTN), a negative regulator of skeletal muscle, causes a remarkable increase in skeletal muscle mass, we posited that MSTN blockade—with MSTN propeptide (MPRO) could improve muscle healing after injury. Our results demonstrated that the injection of an adeno-associated viral (AAV) MPRO vector into normal skeletal muscle, 4 weeks prior to creating a laceration injury in the muscle, led to an improvement of muscle healing when analyzed 4 weeks post-laceration and compared to the control groups. This effect was also observed in long term experimental animals which were sacrificed 1 year post-laceration. Next we explored the potential mechanisms by which MSTN blockade improved skeletal muscle healing. I*n vitro* we observed an enhancement of myoblasts' ability to differentiate into myotubes after AAV-2-MPRO gene transfer. We also demonstrated that muscle progenitor cells (MPCs) isolated from MSTN -/- mice regenerated significantly more myofibers than MPCs isolated from wild type (WT) mice when injected into the dystrophic skeletal muscle of mdx mice. Our results also suggest that MPROs beneficial effects are related to an increase in muscle regeneration, a reduction in fibrosis deposition and an increase in capillary in-growth into the injury site. These results suggest that MSTN blockade has a beneficial effect on muscle healing through an enhancement of the myogenic potential of MPCs as well as through the amelioration of the local environment within the injured skeletal muscle **(Appendices 10 and 12).**

Muscle injuries are very common musculoskeletal problems encountered in sports medicine. Although these injuries are capable of healing, complete functional recovery is hindered by the formation of dense scar tissue triggered by TGF-β1. We have previously reported that several agents such as decorin and suramin which can inhibit fibrosis and improve regeneration in injured skeletal muscle; however, the safety of these agents remains unknown for treating muscle injury. By contrast, Losartan (LOS), one of the Angiotensin II Receptor Blockers (ARBs)—is an FDA approved antihypertensive medication and has been shown to also be antifibrotic in a variety of tissues, including skeletal muscle. This ARB has a well-tolerated side-effect profile, can also block TGF-β1 to attenuate the development of pathological fibrosis. In this study, we investigated optimum doses of LOS for treating injured muscle to help the translation of this research from bench to bedside.
The biphasic effect of LOS on C2C12 myoblasts *in vitro*, stimulating at low dose while decreasing at high dose, suggested that there is an optimal dose of LOS. Consequently we found that LOS improves skeletal muscle regeneration at 4 weeks after contusion injury, except that these effects were reduce/eliminated in 1000 mg/kg/day group. The best effective dose was 300 mg/kg/day. Overall, these effects of LOS were more pronounced for regeneration than for fibrosis. These *in vivo* results are consistent with our *in vitro* results that LOS was able to exert effects on C2C12 whereas fibroblasts were not affected by either ANG or LOS. Regeneration and fibrosis are two competitive processes after muscle injury; therefore, decreasing fibrosis observed in LOS-treated group is can be the result of the increase regeneration. In other words, LOS might indirectly reduced fibrosis by directly stimulating regeneration. Although there were not statistical differences in fibrosis in lower dose LOS groups (3 and 30 mg/kg/day) as compared to control, these effects of LOS on both regeneration and fibrosis showed similar dose dependent trend. However, *in vivo* results above were obtained only from single time point after injury. Since there are time lags between peaks of myofiber regeneration and fibrosis after injury, further investigations are required to examine the effect of LOS on regeneration and fibrosis at their individual peak time, which will facilitate clinical application of ARBs in improving skeletal muscle healing (**Refer to Appendices 8 and 11**).

Angiotensin Receptor Blocker Improves Skeletal Muscle Functional Recovery in a Dose Dependent Manner:

We have previously reported that Losartan (LOS), one of the Angiotensin II Receptor Blockers (ARBs), which is FDA approved for antihypertensive treatment, has been shown to improve muscle healing through antifibrotic action [Bedair HS, *et al*., *Am J Sport Med*. 2008; 36: 1548-54]. We also demonstrated that specific doses of LOS (30 mg/kg/day and higher) improved muscle regeneration and attenuated the development of pathological fibrosis when it was administrated immediately after injury [Uehara K, *et al*., 55th Annual Meeting of the Orthopaedic Research Society, 2009]. In this study, we investigated whether LOS can improve muscle strength recovery after contusion injury, and also attempted to understand the mechanism of LOS action by analyzing gene expression of myostatin and follistatin, which are considered important regulators of skeletal muscle growth.

Improvement of Muscle Strength: LOS improved TA muscle force recovery after contusion injury. Specific peak twitch force and tetanic force was elevated in mice receiving high dose of LOS (30 and 300 mg/kg/day) in comparison to the animals receiving low dose of LOS (3 and 10 mg/kg/day).

Myostatin gene expression: Expression of myostatin in the injured TA muscle in the control-injury and low dose of LOS treatment groups was lower than in the normal TA muscle, while there was no difference between normal and high dose of LOS treatment groups. Expression of follistatin in the control group was higher than in normal muscle and low dose of LOS treatment groups. The highest expression of follistatin was observed in the 30 mg/kg/day of LOS treatment group and it was significantly higher compared to the control group.

Functional recovery is the most important factor in the skeletal muscle healing after injury. Here we demonstrate that LOS administration immediately after injury improves recovery of skeletal muscle strength. These results also support our previous histological findings [Uehara K, *et al*., 55th Annual Meeting of the Orthopaedic Research Society, 2009]. We believe that the mechanism of muscle regeneration after injury might

be related to the expression of follistatin, positive regulator of skeletal muscle growth. We noticed over expression of follistatin in the 30 mg/kg/day treatment group compared to the normal and control-injury groups. These findings correlate with the results of physiological testing. It is unclear why the 30 mg/kg/day LOS displayed an increased expression of myostatin which is a negative regulator of skeletal muscle growth. Our results suggest that continuous administration of the high dose of LOS, in particularly 30 mg/kg/day, immediately after skeletal muscle injury could accelerate skeletal muscle functional recovery. We aimed to evaluate only a single time point of LOS administration immediately after injury. Further studies are required to determine the biological effect of LOS and facilitate the clinical application of ARBs for improvement of skeletal muscle healing **(Appendix 13).**

Angiotensin II Receptor Blocker Ameliorates Skeletal Muscle Healing:

Our previous study revealed that 30 mg/kg/day of LOS treatment was effective in promoting muscle healing and inducing an antifibrotic effect in a murine model of skeletal muscle after injury [Bedair HS, *et al*., *Am J Sport Med*. 2008; 36: 1548-54; Uehara K, *et al*., 55th Annual Meeting of the Orthopaedic Research Society, 2009] . However, the effective dose (30 mg/kg/day) which was administrated immediately after muscle injury is higher compared to the dose used in humans (10 mg/kg/day). In this study we investigated the effect of muscle healing in a murine animal model using the recommended human dose of LOS (10 mg/kg/day) administered at different time points after injury.

LOS enhanced muscle regeneration and reduced fibrosis: We observed significant increases in the number of centronucleated myofibers in the day 3 treatment group when compared with other treatment groups The highest effect on muscle regeneration coincided with significant decrease of fibrosis in the day 3 treatment group.

LOS enhanced muscle force: LOS improved muscle strength recovery after contusion injury. Specific peak twitch force and peak tetanic force ws significantly greater in mice treated with LOS beginning at day 3 after injury.

LOS enhanced expression of Follistatin and Myostatin: Expression of FSTN detected by RT-PCR in the day 3 and day 14 LOS treatment groups was greater than in the normal non-injured or LOS treated at day 0 and day 7 groups. Expression of MSTN in the day 3, day 7 and day 14 LOS treatment groups was lower than in the normal, control and day 0 LOS treatment groups. The highest expression of FSTN coincided with the lowest expression of MSTN in the day 3 LOS treatment group.

This study revealed that the most effective timing for administration of human dose of LOS (10 mg/kg/day) was 3 days after muscle injury. We observed an increased number of centronucleated myofibers and decreased area of fibrosis when LOS was administered at day 3 after injury. The functional recovery after skeletal muscle injury is the most important factor for clinical translation of this therapy. We demonstrate that an enhancement of muscle strength in the day 3 LOS treatment group correlates with the improvement of muscle regeneration and the reduction of fibrosis. We have previously reported that muscle regeneration and fibrosis formation are two concomitant processes after muscle injury, and the effect of LOS was more prominent on muscle regeneration than on fibrosis [Uehara K, *et al*., 55th Annual Meeting of the Orthopaedic Research Society, 2009]. This current study supports this finding. In addition, it may suggest that the administration of LOS effectively leads to enhanced muscle regeneration after muscle injury via the down regulation of endogenous MSTN, which is a negative regulator of skeletal muscle growth. In summary, we indicate that 10 mg/kg/day (human safety dose) of LOS treatment initiated at 3 days after contusion injury can enhance structural and functional healing in mouse skeletal muscle. (**Appendix 14**)

Improving Recovery Following Recurrent Hamstring Injury Using an Angiotensin II Receptor Blocker: Two Case Studies:

Note that the latter findings are provided only as translational evidence of the research being performed at the SCRC. The following studies were funded by the department of Orthopaedic Surgery at the University of

Pittsburgh. Losartan was given to the patient volunteers as an off label use of the drug. No DOD funding was used for these studies.

Given that Losartan has already been used clinically with an extremely safe side effect profile, physicians at the University of Pittsburgh's Department of Orthopaedic Surgery have conducted two case studies in young college athletes that sustained recurrent hamstring injuries and whose recoveries were safely improved with losartan. This is an off-label use of losartan (i.e.: the FDA has not approved labeling the device for the described purpose). Here we report the results obtained.

Subjects: Subject #1: male, 21 years old, college athlete (football punter). He presented 10 days after an acute onset of ~~–s~~earing" pain in his left posterior thigh when he was kicking with his left leg. He referred a similar injury 5 weeks prior to the present injury. Subject #2: male, 22 years old, college athlete (Ultimate Frisbee). He presented 4 days after an acute onset of pain in his left posterior thigh while he was sprinting. He referred two previous hamstring injuries (2 and 7 months prior to the present injury).

MRI results (at time of injury): Subject #1: Acute Grade 2 hamstring strain was observed with a partial thickness tear of the biceps femoris at the proximal myotendinous junction with surrounding edema without an associated avulsion fracture or hematoma. Subject #2: Grade 2 strain with partial thickness tear of the left biceps femoris at the mid aspect, extends approximately 6 cm in the craniocaudal dimension.

*Hamstring flexibility and strength**:*** Subject #1: By the third week after the injury, no deficit was evident in hamstring flexibility. By the ninth week, the isometric hamstring strength measurements at 30 and 90 degrees of knee flexion were 92 and 84% than the uninjured side respectively (Fig.1). Subject #2: Also, by the third week after the injury, no deficit was evident in hamstring flexibility. By the ninth week, the injured side had a higher isometric hamstring strength measurement at 30 and 90 degrees of knee flexion. They were 132% and 110% than the uninjured side respectively.

We have described use of losartan, which is an FDA-approved angiotensin II receptor blocker, to treat two healthy collegiate athletes with a grade 2 biceps femoris injury. The patients tolerated the course of losartan well with no hypotension or any other side effects. Additionally, the patients demonstrated recovery of normal flexibility and strength compared to the contra-lateral leg. Both subjects were ready for return to sports in 9 to 11 weeks after injury (**Appendix 15**).

c) Results of Continuation Objectives (4-1-11 to 12-31-11):

Characterization of Losartan's Mechanism of Action for Muscle Healing

Muscle injuries are a very common musculoskeletal problem encountered in sports medicine. We have previously reported that Losartan (LOS), one of the Angiotensin II Receptor Blockers, when administered at a clinically equivalent dose for the treatment of hypertension in humans (10 mg/kg/day) 3 days after contusion injury, can accelerate muscle healing due to its antifibrotic effect on injured skeletal muscle [1]. LOS is an FDA approved antihypertensive medication and has a well-tolerated side-effect profile; however, its mechanism of action at the clinical dose on the muscle healing process remains unclear. The angiotensin II type 1 receptor (AT1) regulates the expression of myostatin (MSTN) which is a primary negative regulator of muscle growth and a strong stimulator of fibrosis formation, inflammation [2, 3] (figure 1). We hypothesized that regulating the expression of AT1 during the inflammation phase could accelerate muscle healing after skeletal muscle injury by balancing the beneficial aspects of the inflammation process. In this study, we investigated the expression of AT1, MSTN and MyoD (a regulator of muscle regeneration [4]) at different time points after contusion injury and at different initiation times of LOS administration (Day 0 group LOS started at the time of injury, Day 3 LOS started 3 days after injury). Our findings in this study support our previously reported results that showed increased myofiber regeneration and physiological muscle force when LOS is administered 3 days after injury verses no treatment or immediate administration of LOS post-injury. We therefore posit that the mechanism by which LOS accelerates the muscle healing is through the limited or later stage reduction of AT1 during the inflammation process after injury. **(Appendices 18 and 19)**

The Timing of Administration of a Clinically Relevant Dose of Losartan Influences the Healing Process after the Induction of a Muscle Contusion Injury.

Losartan (LOS) is an FDA approved antihypertensive medication that has a well-tolerated side-effect profile. Our previous study revealed that the immediate treatment of a muscle injury with LOS was effective at promoting muscle healing and inducing an antifibrotic effect in a murine model of skeletal muscle injury. In the current study we first investigated the minimum effective dose of administering LOS immediately after injury and subsequently determined whether the timing of administration, of a clinically relevant dose of losartan, would influence its effectiveness for improving muscle healing after a contusion injury. In the first study, the mice were administered with 3, 10, 30 or 300mg/kg/day of LOS immediately after injury and the healing process was evaluated at 2 and 4 weeks post-injury. In the second study, 10mg/kg/day was administered immediately or at 3 or 7 days after injury and the healing process was then evaluated as described above. At 4 weeks post-injury, we observed a significant increase in muscle regeneration and a significant decrease in fibrosis, which consequently led to an improvement in muscle force in the 30 and 300mg/kg/day groups, when LOS was administered immediately following injury. We also observed a significant improvement in muscle healing at 4 weeks post-injury, when the clinically relevant dose of 10mg/kg/day was administered at 3 or 7 days after injury. Our study revealed accelerated muscle healing when the 30mg/kg/day and 300mg/kg/day of LOS was administered immediately after injury and when the clinically relevant dose of 10mg/kg/day of LOS was administered at 3 or 7 days post-injury. (**Appendix 20**)

The combined use of both Losartan and Platelet Rich Plasma to treat muscle contusion injuries

Although muscle contusions are capable of healing, incomplete functional recovery often occurs. We have previously reported that when a safe human equivalent dose of losartan (10mg/kg/day) - one of the FDA approved Angiotensin II Receptor Blockers (ARBs) and an anti-fibrotic agent that blocks TGF-β1 - was administrated 3 days after injury it could promote functional improvement, muscle regeneration and decrease fibrosis at 4 weeks after injury [Bedair HS, et al. 2008]. Moreover, some reports have shown that Platelet-Rich Plasma (PRP), which includes many kinds of growth factors, including TGF-β1, can accelerate muscle healing after injury [Wright-Carpenter T, et al. 2004]. Our hypothesis is that losartan treatment along with the use of PRP can further accelerate the muscle healing process compared to the use of losartan or PRP treatment alone. The purpose of this study was to investigate the potential functional improvement of contusion injured skeletal muscle in mice using both losartan and PRP in combination.

Combining losartan and PRP treatment, following a contusion injury, accelerated skeletal muscle healing. We observed a larger number of regenerating myofibers, enhanced angiogenesis, less fibrosis, and better functional recovery in the PRP/losartan group. These results suggest that PRP/losartan combinatorial treatment after skeletal muscle injury could be more effective than the individual treatments alone and that the beneficial effect of combining PRP and losartan is likely related to the inactivation of TGF-β1 within the PRP by losartan. (**Appendices 21 and 22**)

Key Research Accomplishment

- Determined that Suramin can stimulate MDSC myogenic differentiation *in vitro.*
- Determined that Suramin can enhance skeletal muscle regeneration and reduce fibrous scar formation following a contusion injury.
- Determined that Suramin's beneficial effects on muscle healing is related to its ability to enhance the proliferation and differentiation of myoblasts and MDSC and its ability to inhibit myostatin and up-regulate follistatin.

- Determined that the antifibrotic ability of decorin not only relates to its ability to inhibit TGF-β1 but also myostatin. We further showed that the up or down regulation of TGF-β1 simultaneously had the same effect on myostatin and vice versa.

- Demonstrated that macrophages, TGF- β1, and the COX-2 pathway products may regulate one another's levels and have profound influence on the whole muscle healing process.

- Myostatin has been shown to be a negative regulator of skeletal muscle regeneration and a positive regulator of fibrosis deposition which can be down-regulated with decorin and suramin

- Follistatin is intricately involved with the muscle healing process and has been shown to be a positive regulator of muscle regeneration and a negative regulator of fibrosis deposition and can be up-regulated with decorin and suramin.

- Losartan has been shown to block TGF-β1 and have a statistically significant effect on the healing of injured skeletal muscle.

- Losartan is a safe and effective, clinically applicable angiotensin II receptor blocker and has been shown to be a potential safe and effective treatment for aiding in the healing of skeletal muscle injury.

- 1st case report of treating a recurrent hamstring injury in a 21 year old man with Losatrin is being prepared.

- The continuous administration of Losartan at markedly high dose of 30 mg/kg/day, immediately after skeletal muscle injury, can accelerate skeletal muscle functional recovery.

- 10 mg/kg/day (human safety dose) of LOS treatment initiated at 3 days after contusion injury can enhance structural and functional healing in mouse skeletal muscle.

- Follistatin stimulates myoblasts to express MyoD, Myf5, and myogenin, which are myogenic regulatory factors that promote the myogenic differentiation of myoblasts into myotubes.

- Follistatin induces differentiation through the inhibition of myostatin, activin A and transforming growth factor – beta 1, which are negative regulators of myoblast differentiation.

- MSTN blockade via MPRO has a beneficial effect on muscle healing through an enhancement of the myogenic potential of MPCs as well as through the amelioration of the local environment within the injured skeletal muscle.

- MPRO's beneficial effects are related to an increase in muscle regeneration, a reduction in fibrosis deposition and an increase in capillary in-growth into the injury site.

- Losartan was used to treat two healthy collegiate athletes with grade 2 biceps femoris injuries and tolerated its off-label use well with no hypotension or any other side effects. The patients demonstrated recovery of normal flexibility and strength compared to the contra-lateral leg and were capable of returning to sports activity in 9 to 11 weeks after injury.

- The combinatorial use of both PRP injection snd oral administration of losartan significantly improved the functional outcome of contusion injured skeletal muscle in mice compared to either treatment alone.

- Accelerated muscle healing is observed when the 30mg/kg/day and 300mg/kg/day of LOS was administered immediately after injury and when the clinically relevant dose of 10mg/kg/day of LOS was administered at 3 or 7 days post-injury.

- The angiotensin II type 1 receptor (AT1) regulates the expression of myostatin (MSTN) which is a primary negative regulator of muscle growth and a strong stimulator of fibrosis formation and inflammation. Regulation of the angiotensin II type 1 receptor (AT1) which regulates the expression of MSTN, a negative regulator of muscle growth, with losartan can reduce fibrosis and increase muscle regeneration, hence regulating the expression of AT1 with losartan during the inflammation phase could accelerate muscle healing after skeletal muscle injury by balancing the beneficial aspects of the initial onset of the inflammation process.

Reportable Outcomes

Nozaki M, Li Y, Zhu J, Ambrosio F, Fu FH and Huard J. The use of suramin to improve skeletal muscle healing after contusion injury. Presented as a poster at the 53rd Annual Meeting; San Diego, CA; Feb 12-14, 2007. **(Appendix 1)**

Li Y, Li J, Zhu J, Sun B, Branca M, Tang Y, Foster W, Xiao X, Huard J. Decorin gene transfer promotes muscle cell differentiation and muscle regeneration. Mol Ther 2007 Sep; 15(9):1616-1622. **(Appendix 2)**

Zhu J, Li Y, Shen W, Qiao C, Ambrosio F, Lavasani M, Nozaki M, Branca M, Huard J. Relationships between TGF-β1, myostatin, and decorin: Implications for skeletal muscle fibrosis. J Biol Chem 2007 Aug; 282(35):25852-63. **(Appendix 3)**

Zhu J, Li Y, Huard J. Decorin Interacts with Myostatin Activity-Implications for Skeletal Muscle Healing. The Orthopaedic Research Society (ORS) 53rd Annual Meeting; San Diego, CA; Feb 12-14, 2007. **(Appendix 4)**

Zhu J, Li Y, Branca M, Huard J. Follistatin improves skeletal muscle healing after injury. The Orthopaedic Research Society (ORS) 53rd Annual Meeting; San Diego, CA; Feb 12-14, 2007. **(Appendix 5)**

Shen W, Huard J. Tissue therapy: Implications of regenerative medicine for skeletal muscle. Attala A, Lanza R, Thomson J, Nerem RM, editors. Principles of Regenerative Medicine. Elsevier Academic Press, 2007; 1232--1247, chapter 72. (Invited Book Chapter)

Matsuura T, Li Y, Giacobino JP, Fu F, Huard J. Skeletal muscle fiber type conversion during the repair of mouse soleus: Potential implications for muscle healing after injury. J. Orthop Res 2007 Nov; 25(11): 1534-1540**.** Figure featured on front cover as cover art.

Nozaki M, Li Y, Zhu J, Uehara K, Ambrosio F, Fu F, Huard J. Suramin can enhance the skeletal muscle healing by blocking myostation. The Orthopaedic Research Society (ORS) 54th Annual Meeting; March 2-5, 2008; San Francisco, CA. **(Appendix 6)**

Nozaki M, Li Y, Zhu J, Ambrosio F, Uehara K, Fu F, Huard J. Improved muscle healing after contusion injury by the inhibitory effect of Suramin on Myostatin, a negative regulator of muscle growth. Am J Sports Med, 2008 Dec; 36(12):2354-62. **(Appendix 7)**

Bedair H, Karthikeyan T, Quintero AJ, Li Y, Huard J. Angiotensin II Receptor Blockade administered after injury improves muscle regeneration and decreases fibrosis in normal skeletal muscle. Am J Sports Med, 2008 Aug; (36)8: 1548-1555. **(Appendix 8)**

Shen W, Li Y, Zhu J, Schwendener R, Huard J. Interaction between macrophages, TGF-beta 1, and the COX-2 pathway during the inflammatory phase of skeletal healing after injury. J of Cellular Physiology 2008 Feb; 214(2): 405-412. **(Appendix 9)**

Ambrosio, F, Li Y, Usas A, Boninger, ML, Huard J. Muscle repair after injury and disease. Orthopaedic Biology and Medicine, Musculoskeletal Tissue Regeneration: Biological Materials and Methods. The Humana Press Inc, 2008. (Invited Review)

Zhu J, Ma J, Lu A, Qiao C, Li J, Li Y, Xiao X, Huard J. Blocking Myostatin by AAV2-Delivered Myostatin Propeptide Improves Muscle Cell Transplantation **.** Orthopeadic Research Society; Las Vegas, Nevada; February 22-25, 2009. **(Appendix 10)**

Uehara K, Nozaki M, Zhu J, Quintero A, Ota S, Fu F, Huard J. Angiotensin II Receptor Blocker Ameliorates Skeletal Muscle Healing in a Dose Dependent Manner. 55th Annual Orthopeadic Research Society Meeting; Las Vegas, Nevada; February 22-25, 2009. **(Appendix 11)**

Zhu J, Ma J, Qiao C, Jianbin L, Yong L, Xiao X, Huard J. Blocking Myostatin Improves Muscle Healing Via Enhancement of Angiogenesis. 55th Annual Orthopeadic Research Society Meeting; Las Vegas, Nevada; February 22-25, 2009. **(Appendix 12)**

Uehara, K; Kobayashi, T; Ota, S; Bin, S; Tobita, K; Ambrosio, F; Fu, FH; Huard, J. Angiotensin Receptor Blocker Improves Skeletal Muscle Function Recovery in a Dose Dependent Manner. 56th Annual Orthopeadic Research Society Meeting; New Orleans, LA; March 6-9, 2010. **(Appendix 13)**

Kobayashi, T; Uehara, K; Ota, S; Bin, S; Tobita, K; Ambrosio, F; Fu, FH; Huard, J. Angiotensin II Receptor Blocker Ameliorates Skeletal Muscle Healing, 56th Annual Orthopeadic Research Society Meeting; New Orleans, LA; March 6-9, 2010.**(Appendix 14)**

Yuri Chun MD, Sheila J M Ingham MD, James Irrgang PhD, Tanya Hagen MD, Freddie Fu MD, Burhan Gharaibeh, Vonda Wright MD, Johnny Huard, PhD. Improving Recovery Following Recurrent Hamstring Injury Using an Angiotensin II Receptor Blocker: Two Case Studies; 56th Annual Orthopeadic Research Society Meeting; New Orleans, LA; March 6-9, 2010. **(Appendix 15)**

Jinhong Zhu, M.D., Ph.D., Yong Li, M.D., Ph.D., Aiping Lu, MD., Burhan Gharaibeh, Ph.D., Jianqun Ma, M.D., Ph.D., Andres J. Quintero, M.D., Tetsuo Kobayashi, M.D., Johnny Huard, Ph.D., Follistatin Improves Skeletal Muscle Healing by Blocking TGF-β-like Signaling Pathway. (Am Jour of Path. 2011 Aug; 179(2):915-30.) **(Appendix 16)**

Terada S, Kobayashi T, Ota S, Gharaibeh B, Fu FH, Huard J. Angiotensin II Receptor Blocker and Muscle Derived Stem Cells Transplantation Treatment for Contusion Skeletal Muscle Injury in Mice. 2011 ORS Annual Meeting; January 13-16, 2011; Long Beach, CA. (**Appendix 17)**

Kobayashi, T; Uehara, K;Ota, S; Terada, S; Cummins, JH; Fu, FH; Huard, J. Characterization of Losartan's Mechanism of Action for Muscle Healing. 2011 Annual Orthopeadic Research Society Meeting;; January 13-16, 2011; Long Beach, CA (**Appendix 18)**

Kobayashi T, Ota S, Uehara K, Terada S, Tobita K, Ambrosio F, Fu F, Huard J. Angiotensin II Receptor Blocker Promote Muscle Healing After Injury Through Activation of Muscle Regulations. 8th Biennial ISAKOS; May 15 -19, 2011; Rio de Janeiro, Brazil (**Appendix 19**)

Kobayashi T, Uehara K, Ota S, Tobita K, Ambrosio F, Cummins J, Fu F, Huard J. The Effect of Timing of Administration of Losartan on Skeletal Muscle Healing After Traumatic Injury. AFIRM All Hands meeting; Pete, FL; January 17-20, 2011.

Uehara K, Ota S, Nozaki M, Kobayashi T, Tobita K, Ambrosio F, Fu F, Huard J. Angiotensin II Receptor Blocker Ameliorates Skeletal Muscle Healing through Dose Dependent Manner. AFIRM All Hands meeting; Pete, FL; January 17-20, 2011.

Tetsuo Kobayashi, Kenji Uehara, Shusuke Ota, Kimimasa Tobita, Fabrisia Ambrosio, James H. Cummins, Satoshi Terada, Freddie H. Fu and Johnny Huard. The Timing of Administration of a Clinically Relevant Dose of Losartan Influences the Healing Process after the Induction of a Muscle Contusion Injury. (Under 2nd Revision in J. of Applied Physiology). (**Appendix 20**)

Combination Treatment of Platelet-Rich Plasma and Angiotensin II Receptor Blocker for Contusion Skeletal Muscle Injury in Mice . Satoshi Terada; Shusuke Ota; Tetsuo Kobayashi; Yutaka Mifune; Koji Takayama; Sahnghoon Lee; Burhan Gharaibeh; Takanobu Otsuka, Freddie H. Fu and Johnny Huard. 2011 Annual Orthopeadic Research Society Meeting; February 4-7, 2012, San Francisco, CA. (**Appendix 21)**

The use of an Anti-Fibrotic Agent Improves the Effect of Platelet Rich Plasma on Muscle Healing after Injury. Satoshi Terada, MD, Shusuke Ota, MD, PhD, Tetsuo Kobayashi, MD, PhD, Yutaka Mifune, MD, PhD, Koji Takayama, MD, PhD, Sahnghoon Lee MD, PhD, Michelle Witt, MS ,Burhan Gharaibeh, PhD,Gianluca Vadala, MD, Takanobu Otsuka, MD, PhD, Freddie H. Fu, MD, and Johnny Huard, PhD. (In Submission to the Am J of Bone and Joint Surgery) (**Appendix 22**)

Burhan Gharaibeh, Yuri Chun, Tanya Hagen, Sheila McNeill Ingham, Vonda Wright, Freddie Fu and Johnny Huard. Biological approaches to improve skeletal muscle healing after injury and disease. Embryology Today, Review Article (In Press) (**Appendix 23**)

Conclusions

This line of work has enabled us to identify three potentially useful compounds that could be utilized to prevent fibrous scar formation and aid in the healing and regeneration of injured skeletal muscle. Suramin is a drug that has been used clinically to treat individuals infected with trypanosomes and worms and is currently being investigated for the treatment of prostate cancer; therefore it could potentially be applied clinically and expeditiously. Decorin too has shown great promise; however, it is not currently FDA approved for clinical application and would require more time to apply clinically since it would need to undergo clinical trials. From a basic science standpoint these studies have demonstrated the mechanisms that these compounds utilize for their beneficial effect on the healing and regeneration of skeletal muscle. They firstly act by inhibiting two components that are major initiators of the fibrosis cascade, TGF- β1 and myostatin. They have also been shown to facilitate regeneration and healing by promoting myoblast and MDSC proliferation and differentiation

into myotubes in vitro and myofibers in vivo. One of the pathways that they seem to work on for this enhancement in regeneration appears to be the up-regulation of follistatin. Additional work is currently underway to determine the pharmokinetics of these two promising compounds. We have been exploring the use of the angiotensin II receptor blocker, Losartan, and have reported a case study that follows 2 patients and demonstrates that this drug was well tolerated by these 2 patients who were suffering from recurrent hamstring injuries. Additional animal studies are underway to explore the molecular mechanisms involved with the successful use of this drug to treat skeletal muscle injuries besides its ability to block TGF-β1. We have also been performing experiments with the myostain propepdtide (MPRO) which blocks the action of myostatin by directly binding to the molecule and hence inhibiting the fibrosis cascade from initiating. We have also shown that MPRO has a muscle regenerative action as well which has been shown to be related to the stimulation of angiogenesis in the injured muscle. Finally, we have explored the use of follistatin to inhibit fibrosis formation and increase muscle regeneration in injured skeletal muscle. We demonstrated that follistain blocks 3 major negative regulators of muscle growth including myostatin, TGF-beta1 and activin A. We also showed that follistatin can stimulate the myogenic differentiation of myoblasts which promotes muscle regeneration.

Appendices

Appendix 1: Nozaki M, Li Y, Zhu J, Ambrosio F, Fu FH and Huard J. The use of suramin to improve skeletal muscle healing after contusion injury. Presented as a poster at the Orthopaedic Research Society meeting, Feb. 2007.

Appendix 2: Li Y, Li J, Zhu J, Sun B, Branca M, Tang Y, Foster W, Xiao X, Huard J. Decorin gene transfer promotes muscle cell differentiation and muscle regeneration. Mol Ther 2007 Sep; 15(9):1616-1622.

Appendix 3: Zhu J, Li Y, Shen W, Qiao C, Ambrosio F, Lavasani M, Nozaki M, Branca M, Huard J. Relationships between TGF-β1, myostatin, and decorin: Implications for skeletal muscle fibrosis. J Biol Chem 2007 Aug; 282(35):25852-63.

Appendix 4: Zhu J, Li Y, Huard J. Decorin Interacts with Myostatin Activity-Implications for Skeletal Muscle Healing. The Orthopaedic Research Society (ORS) 53rd Annual Meeting; San Diego, CA; Feb 12-14, 2007.

Appendix 5: Zhu J, Li Y, Branca M, Huard J. Follistatin improves skeletal muscle healing after injury. The Orthopaedic Research Society (ORS) 53rd Annual Meeting; San Diego, CA; Feb 12-14, 2007.

Appendix 6: Nozaki M, Li Y, Zhu J, Uehara K, Ambrosio F, Fu F, Huard J. Suramin can enhance the skeletal muscle healing by blocking myostation. The Orthopaedic Research Society (ORS) 54th Annual Meeting; San Francisco, CA; March 2-5, 2008.

Appendix 7: Nozaki M, Li Y, Zhu J, Ambrosio F, Uehara K, Fu F, Huard J. Improved muscle healing after contusion injury by the inhibitory effect of Suramin on Myostatin, a negative regulator of muscle growth. Am J Sports Med, 2008 Dec; 36(12):2354-62.

Appendix 8: Bedair H, Karthikeyan T, Quintero AJ, Li Y, Huard J. Angiotensin II Receptor Blockade administered after injury improves muscle regeneration and decreases fibrosis in normal skeletal muscle. Am J Sports Med, 2008 Aug; (36)8: 1548-1555.

Appendix 9: Shen W, Li Y, Zhu J, Schwendener R, Huard J. Interaction between macrophages, TGF-beta 1, and the COX-2 pathway during the inflammatory phase of skeletal healing after injury. J of Cellular Physiology 2008 Feb; 214(2): 405-412.

Appendix 10: Zhu J, Ma J, Lu A, Qiao C, Li J, Li Y, Xiao X, Huard J. Blocking Myostatin by AAV2-Delivered Myostatin Propeptide Improves Muscle Cell Transplantation**.** Orthopeadic Research Society; Las Vegas, Nevada; February 22-25, 2009.

Appendix 11: Uehara K, Nozaki M, Zhu J, Quintero A, Ota S, Fu F, Huard J. Angiotensin II Receptor Blocker Ameliorates Skeletal Muscle Healing in a Dose Dependent Manner. Orthopeadic Research Society; Las Vegas, Nevada; February 22-25, 2009.

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Appendix 14: Kobayashi, T; Uehara, K; Ota, S; Bin, S; Tobita, K; Ambrosio, F; Fu, FH; Huard, J. Angiotensin II Receptor Blocker Ameliorates Skeletal Muscle Healing, 56th Annual Orthopeadic Research Society Meeting; New Orleans, LA; March 6-9, 2010.

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Appendix 22: Satoshi Terada; Shusuke Ota; Tetsuo Kobayashi; Yutaka Mifune; Koji Takayama; Sahnghoon Lee; Burhan Gharaibeh; Takanobu Otsuka, Freddie H. Fu and Johnny Huard. Combination Treatment of Platelet-Rich Plasma and Angiotensin II Receptor Blocker for Contusion Skeletal Muscle Injury in Mice . 2011 Annual Orthopeadic Research Society Meeting; February 4-7, 2012, San Francisco, CA.

Appendix 23: Satoshi Terada, MD, Shusuke Ota, MD, PhD, Tetsuo Kobayashi, MD, PhD, Yutaka Mifune, MD, PhD, Koji Takayama, MD, PhD, Sahnghoon Lee MD, PhD, Michelle Witt, MS ,Burhan Gharaibeh, PhD,Gianluca Vadala, MD, Takanobu Otsuka, MD, PhD, Freddie H. Fu, MD, and Johnny Huard, PhD. The use of an Anti-Fibrotic Agent Improves the Effect of Platelet Rich Plasma on Muscle Healing after Injury. (In Submission to the Am J of Bone and Joint Surgery)

Appendix 24: Burhan Gharaibeh, Yuri Chun, Tanya Hagen, Sheila McNeill Ingham, Vonda Wright, Freddie Fu and Johnny Huard. Biological approaches to improve skeletal muscle healing after injury and disease. Embryology Today, Review Article (In Press)

Project # 2 Final Report
Improving muscle healing through digestion of scar tissue via MMP-1
(Yong Li)
****Note that this subproject was completed last February 2011. Below is a reiteration of last year's final report**

Introduction:

Muscle injuries, which occur most frequently during sports activity, military training and battle; present a challenging problem in traumatology. After injury, damaged muscle fibers undergo a natural process of necrosis and the resulting dead tissue is removed by infiltrating lymphocytes. Meanwhile, locally released growth factors stimulate muscle regeneration by activating satellite cells. Unfortunately, the process of muscle regeneration is often incomplete from overgrowth of the extracellular matrix (ECM) leading to significant local fibrosis (i.e., fibrous scar formation). This scar tissue impedes the formation of normal muscle fibers in the injured muscle, resulting in incomplete functional recovery and a propensity for re-injury. We have begun to study the mechanism behind the fibrosis that occurs in injured skeletal muscle. Our previous studies have demonstrated that myogenic cells (including muscle-derived stem cells [MDSCs]) and regenerating myofibers in lacerated muscle can differentiate into fibrotic cells, and that transforming growth factor (TGF)-beta1 is a major stimulator of this differentiation. Using different animal models of muscle injury, we have investigated biological approaches by which to prevent fibrosis and thereby improve muscle healing. However, it often is not possible to treat injured muscles before the initiation of fibrosis—most patients with muscle injuries seek treatment only after the onset of fibrous scar formation, and the concomitant pain and functional deficits it produces. Moreover, chronic diseases (e.g., Duchenne muscular dystrophy [DMD]) generally present significant amounts of fibrous scar tissue within the patients' muscles. Because prevention of fibrosis is infeasible in many cases, the development of a novel therapeutic approach by which to digest existing fibrous scar tissue and improve muscle healing would be very significant.

Matrix metalloproteinase type-1 (MMP1), a naturally occurring collagen-digesting enzyme, has shown great capacity for digesting fibrous scar formations in various tissues. Additionally, MMP1 is also able to increase cell migration in many tissues. We believe that MMP1 is able to facilitate the healing of injured muscle by digesting fibrous scar tissue and improving the local environment in which muscle regeneration occurs. Results obtained from this project may lead to the development of gene therapy applications that eliminate scar tissue within skeletal muscle. Such applications could drastically improve the regeneration of muscles damaged by trauma or by chronic muscle diseases, such as Duchenne and Becker muscular dystrophies.

In the past few years, we have completed most of the proposed experiments. Through our work on **Objective #1**, we have determined that MMP1 increased muscle cell migration and differentiation/fusion capacity in vitro. In vivo, we also determined that MMP1 could improve muscle healing through increasing muscle cell migration, differentiation and regeneration (**Objective #2**). Finally, we have discovered that MMP1 gene transfer enhanced muscle cell migration and differentiation in vitro and in vivo (**Objective #3**).

Body:

Progress to date (3-3-06 to 2-28-10):

Technical Objective #1: To assess the effect of MMP1 on muscle cells *in vitro*.

This proposed experiment has been completed in the past two years. Results from this objective indicate that MMP1 is able to inhibit fibroblast growth and collagen deposition as well as enhance myoblasts migration and fusion/differentiation of these cells *in vitro*. The completed experiment has carried out two publications in the *Journal of Applied Physiology* [2007;102(6):2338-2345] and *American Journal of Pathology* [2009;174(2);541-549] and three review articles: *Future Medical Chemistry* [2009;1(6):1095-1111], *Cell Adhesion & Migration* [2009;3(4):337-341]. *US Musculoskeletal Review*, [2009; 4 (1):74-77].

Technical Objective #2: To assess the effects of MMP1 on skeletal muscle *in vivo*.

In this objective we observed the effects of MMP1 on skeletal muscle healing in animal models *(in vivo*). We have finished most of this proposed experiment. By using a reproducible muscle injury model in mice that induced significant fibrous scar formation, hindered myofiber regeneration and slowed muscle healing, we have determined that MMP1 can digest the existing fibrous scar and promote muscle healing in the injured skeletal muscles. This proposed experiment has been completed in the past year, and this study has been published by the *Journal of Applied Physiology* 2007;102(6):2338-2345. In addition, to observe the effect of MMP1 on muscle cell migration and differentiation in vivo, we have selected MDX mice, a mouse model for Duchenne muscular dystrophy [DMD], to investigate if MMP1 could enhance muscle cell migration and differentiation in vivo. Our results have substantiated this point and showed that MMP1 could enhance muscle cell fusion as well as migration in dystrophic muscle. This research have been published by *American Journal of Pathology* [2009;174(2);541-549] and have been discussed in our review papers: *Future Medical Chemistry* [2009;1(6):1095-1111], *Cell Adhesion & Migration* [2009;3(4):337-341]. *US Musculoskeletal Review*, [2009; 4 (1):74-77]. (Please see attached copy of manuscript).

Technical Objective #3: To assess the use of MMP1–based gene therapy to digest fibrous scar tissue within injured skeletal muscle.

The primary aim of this proposal is to investigate whether MMP1 gene transduction in implantable myoblasts can extend MMP1 function as a means of digesting fibrous scar tissue and promoting muscle cell migration, fusion and regeneration within injured skeletal muscle. MMP1 can degrade collagens, but undergoes self–degradation, which results in a short biological half-life. As a result, the fibrous scar tissue has the potential to reform following MMP1 degradation. To extend the function of MMP1, we have constructed a retrovirus vector encoding the MMP1 gene. These related studies and results are preparing one paper draft and is pending to send to publish. In the MMP1 gene therapy, we have discovered that #1. Human MMP1 gene expresses in target muscle cells; #2. MMP-1 gene transduction stimulated myoblasts to increase migration. #3. Gene transfer of MMP1 enhances myoblast differentiation *in vitro*. #4. MMP1 gene therapy improves myoblast transplantation in MDX/SCID (a dystrophic/immunodeficient mouse model) mice. #5. MMP1 gene transduction also increased myoblast migration after implantation *in vivo*. #6. Myoblasts genetically engineered to express MMP1 display a strong migration capacity following systemic delivery into *MDX/SCID* mice. These results support the notion that MMP1 is able to increase myoblast migration, differentiation and help injured skeletal muscle regeneration to speed healing process.

Future plans:

We have successfully completed all the proposed objectives. Our future plan will focus on the mechanism investigation of MMPs in muscle healing process and other potential function of MMP treatment in the musculoskeletal system which includes stem cell stimulation, angiogenesis and reinnervations in injured skeletal muscle.

Key Research Accomplishments:

1. Discovery of the effect of MMP1 on muscle cells, resulting in increased migration and differentiation of myogenic cells *in vitro*.
2. Discovery of the effect of MMP1 on scarring skeletal muscle, namely, the digestion of fibrous scar tissue, and improved muscle healing *in vivo*.
3. Discover of the effect of MMP1 on muscle cell migrations and fusion in skeletal muscle of mice *in vivo*.

4. Discovery of MMP1 gene transfer to extend its half-life, which prolongs MMP1 function during its application both *in vitro and in vivo*.

Reportable outcomes:

@ Four manuscripts:

1. Bedair H, Liu TT, Kaar J, Shown B, Russell A, Huard J, Li Y**.** Matrix Metalloproteinase (MMP) Therapy Improves Muscle Healing. ***J Applied Physiology*** 2007;102(6):2338-45.

2. Kaar JL, Li Y, Blair HC, Asche G, Koepsel RR, Huard J, Russell AJ. Matrix metalloproteinase-1 treatment of muscle fibrosis. ***Acta Biomater.*** 2008;4(5):1411-1420.

3. Wang W, Pan HY, Murray K, Jefferson M, Li Y. MMP1 promotes muscle cells migration and differentiation. ***American J Pathology*** 2009, 174 (2); 541-549.

4. Pan HY, Liu T, Wang B, Ikozawa M, Huard J, Li Y. MMP1 gene therapy to enhance muscle cell migration and differentiation. (in preparing)

@ Three review articles:

1. Mu XD, Wang W, Li Y. Myoblast transplantation and fibrosis prevention in diseased muscle (invited review). ***US Musculoskeletal Review*** 2009;4(1):74-77.

2. Bellayr I, Li Y. **Biochemical insights into the role of matrix metalloproteinases in the regeneration: challenges and recent developments.** Future Medicinal Chemistry 2009;1(6):1095-1111.

3**. Chen XP, Li Y. Role of matrix metalloproteinases in skeletal muscle: migration, differentiation, and regeneration.** ***Cell Adhesion & Migration*** 2009;3(4):337-341.

Conclusion:

In vitro, we have seen that MMP1 can enhance muscle cell migration and differentiation. The migration–related proteins are also up–regulated within MMP1–treated muscle cells. *In vivo*, the fibrous scar tissues that form within traumatically injured skeletal muscle could limit transplanted myogenic cell migration, fusion, and regeneration, thus slow the overall muscle healing process. However, muscle healing was greatly improved following MMP1 treatment within these scarred skeletal muscles. We also discovered that the use of MMP1 gene–transferred myoblasts resulted in an increased differentiation capacity *in vitro* and enhanced muscle cell migration *in vivo*.

Appendix and Reference:

1. Bedair H, Liu TT, Kaar J, Shown B, Russell A, Huard J, Li Y**.** Matrix Metalloproteinase (MMP) Therapy Improves Muscle Healing. ***J Applied Physiology*** 2007;102(6):2338-45.

2. Kaar JL, Li Y, Blair HC, Asche G, Koepsel RR, Huard J, Russell AJ. Matrix metalloproteinase-1 treatment of muscle fibrosis. ***Acta Biomater.*** 2008;4(5):1411-1420.

3. Wang W, Pan HY, Murray K, Jefferson M, Li Y. MMP1 promotes muscle cells migration and differentiation. ***American J Pathology*** 2009, 174 (2); 541-549.

4. Mu XD, Wang W, Li Y. Myoblast transplantation and fibrosis prevention in diseased muscle (invited review). ***US Musculoskeletal Review*** 2009;4(1):74-77.

5. Bellayr I, Li Y. **Biochemical insights into the role of matrix metalloproteinases in the regeneration: challenges and recent developments.** Future Medicinal Chemistry 2009;1(6):1095-1111.

6. Chen XP, Li Y. Role of matrix metalloproteinases in skeletal muscle: migration, differentiation, and regeneration. ***Cell Adhesion & Migration*** 2009;3(4):337-341.

7. Pan HY, Liu T, Wang B, Ikozawa M, Huard J, Li Y. MMP1 gene therapy to enhance muscle cell migration and differentiation. ***Molecule Therapy*** (in preparing)

Project # 3 Final Report**
Repairing injured skeletal muscle through myogenic endothelial cells
(Bruno Peault)

****Note that this subproject was completed last February 2009. Below is a reiteration of last year's final report**

INTRODUCTION

Upon muscle injury, satellite cells, the professional myogenic progenitors present in skeletal muscle, can divide, differentiate and fuse to generate myofibers [1, 2]. Injection of myoblasts has been attempted to repair both skeletal and cardiac muscles in animals and humans [3-5] and was to some extent successful but severe limitations were also encountered after myoblast implantation in both tissues[3, 6, 7,]. Muscle regeneration might be improved by transplanting stem cells, in some instances after therapeutic gene transfer, instead of later myogenic progenitors [8-12].

As described in other sections of this report, we have previously isolated, based on their slow adherence in culture, early myogenic progenitor cells, named muscle derived-stem cells (MDSC), that more efficiently regenerate both skeletal and cardiac muscles than satellite cells [9, 13]. MDSC were isolated retrospectively from cultured skeletal muscle; therefore their origin, identity and anatomic location in the native muscle are still unknown. The expression by MDSC of the myogenic cell markers desmin and MyoD, in addition to CD34 and Sca-1 and absence of c-kit and CD45, distinguishes these cells from other known adult stem cells such a MSC and MAPC.

We have, in the present study, investigated the identity and anatomical location of muscle-derived stem cells in human skeletal muscle. Our conclusion is that a previously unexplored developmental relationship exists between endothelial and myogenic cells. Indeed, a subset of MDSC express endothelial cell markers, and MDSC spontaneously differentiate into endothelial cells, and/or promote angiogenesis, probably *via* VEGF secretion, following implantation in skeletal and cardiac muscles [9, 13].

Besides regular satellite cells and endothelial cells, we have identified and characterized a novel population of *myo-endothelial* cells that co-express myogenic and endothelial cell markers. When injected into the injured skeletal muscles of immunodeficient mice, FACS-sorted cells co-expressing myogenic and endothelial cell markers (CD56+CD34+CD144+) regenerated skeletal muscle fibers much more efficiently than conventional CD56+ myogenic cells. Myoendothelial cells clonally expanded from single sorted cells differentiated into myogenic, chondrogenic and osteogenic cells under appropriate culture conditions.

KEY RESEARCH ACCOMPLISHMENTS

We have achieved the molecular identification and sorting of human myo-endothelial cells. Human skeletal muscle sections were immunostained with antibodies directed against myogenic and endothelial cell antigens. A subset of satellite cells (Pax7+ or CD56+) co-express endothelial cell antigens: VE-cadherin (CD144), vWF, the UEA-1 receptor and CD34. The percentages of Pax7+ cells also expressing these endothelial cell antigens were, respectively, 8.9%, 9.5%, 9.5% and 9.8%, as counted on at least 100 satellite cells in five independent experiments. Co-expression of these molecules by individual cells was ascertained by confocal microscopy. These results suggested the existence within adult human skeletal muscle of a rare subset of cells that co-express myogenic and endothelial cell markers. Adult skeletal muscle samples were then analyzed by flow cytometry after enzymatic dissociation. CD45- viable cells were gated and further separated into CD56+ myogenic cells and CD56- non-myogenic cells. As expected, the latter subset of non-myogenic cells contained the bulk of endothelial cells (CD34+CD144+). However, CD56+ cells also contained a very minor subset of cells expressing CD34 and CD144, thus confirming the existence of cells co-expressing markers of the myogenic and vascular endothelial cell lineages. Using the same immunostaining conditions, we then proceeded to sort the above-described cell subsets by FACS. The mean numbers of viable sorted cells which were recovered per experiment were $4.8 \pm 1.3 \times 10^4$ CD56+CD34-CD144- myogenic cells, $7.7 \pm 2.6 \times 10^4$ CD56-CD34+CD144+ endothelial cells and $1.5 \pm 0.8 \times 10^4$ CD56+CD34+CD144+ myoendothelial cells.

Purities of these three sorted cell populations were, respectively, 93.8 ± 1.0 %, 92.2 ± 1.1 % and 93.5 ± 1.8% [14-16]

We have regenerated skeletal mucle with human myoendothelial cells Myoendothelial, endothelial and satellite cells sorted from adult skeletal muscle were injected intramuscularly, in multiple distinct experiments, into SCID mouse skeletal muscles that had been injured by cardiotoxin. After 10 days, mice were sacrificed and skeletal muscles were analyzed by immunohistochemistry using an antibody directed to human spectrin. All three cell categories tested regenerated spectrin-expressing muscle fibers but quantitative analysis revealed that the regenerative potential of myoendothelial cells is by far the highest. Indeed, 1000 myoendothelial cells generated on average 89 myofibers, as compared with 9 and 5 myofibers produced when the same number of cells expressing exclusively endothelial or myogenic cell markers was injected, respectively. These results support our hypothesis that muscle vascular endothelial cells and a novel subset of cells with an overlapping phenotype between myogenic and endothelial cells are endowed with high muscle regeneration potential[14-16]

We have cultured myoendothelial cells over the long term, and shown that these proliferate faster, and survive better under oxidative stress than myogenic cells and endothelial cells Endothelial, myoendothelial and satellite cells were cultured independently in the medium used for MDSC culture[9] for 5 to 6 weeks. All 3 subsets of long-term cultured cells remained capable of differentiating into myotubes in vitro. Cultured cells injected into SCID mouse muscles damaged with cardiotoxin also retained their ability to regenerate myofibers, and myoendothelial cells remained the most efficient myogenic progenitors in these assays.

Myoendothelial cells proliferate significantly faster than the two other cell groups. Having previously observed that the high regenerative capacity of mouse MDSC in skeletal and cardiac muscles is related to their resistance to oxidative stress [13], we treated the three populations under study with hydrogen peroxide in culture. Myoendothelial cells are the most resistant to oxidative stress as indicated by lower levels of cell death under these conditions. [14-16]

We have shown that myoendothelial cells are, at the clonal level, multi-lineage mesodermal stem cells We investigated the multipotency of myoendothelial cells, isolated from adult human skeletal muscle, at the single-cell level. Myoendothelial cell clones express markers of myogenic, endothelial, perivascular and mesenchymal stem cells (MSCs). These clonal cells can be differentiated into endothelial cells, smooth muscle cells, pericytes, adipocytes, chondrocytes and osteoblasts *in vitro* and participated in vessel, bone and cartilage regeneration *in vivo*. We therefore demonstrate that human myoendothelial cells possess some fundamental properties of stem cells.[17]

REPORTABLE OUTCOMES

- Peault, B. et al. Stem and Progenitor Cells in Skeletal Muscle Development, Maintenance, and Therapy. *Mol Ther* (2007)
- Zheng, B., Cao, B., Crisan, M., Sun, B., Li, G.H., Logar, A., Yap, S., Pollett, J.B., Drowley, L., Cassino, T., Gharaibeh, B., Deasy, B., Huard, J., Péault, B. Prospective identification of myogenic endothelial cells in human skeletal muscle. *Nature Biotechnology*, 25(9):1025-1034 (2007
- Crisan, M., Deasy, B., Gavina, M., Zheng, B., Huard, J., Lazzari, L., Péault, B. Purification and long-term culture of multipotent progenitor cells affiliated with the walls of human blood vessels: myoendothelial cells and pericytes. *Methods In Cell Biology*, 86:295-309. (2008)
- Crisan, M., Zheng, B., Sun, B., Yap, S., Logar, A., Huard, J., Giacobino, J.P., Casteilla, L., Péault, B. Purification and culture of human blood vessel-associated progenitor cells. Current Methods in Stem Cell Biology. In Press.
- Zheng, B., Li,G., Deasy, B., Pollett. J., Sun, B, Drowley, L., Gharabeh, B., Usas, A., Logar, A, Peault, B., and Huard, J. The myoendothelial cell: a novel stem cell from adult human skeletal muscle. *In preparation*

Although the present project has been focused on the regeneration of *skeletal* muscle, we have also documented the strong potential of these cells to regenerate the myocardium and improve cardiac function post-infarction:

- Okada, M., Payne, T., Zheng, B., Oshima, H., Momoi, N., Tobita, K., Keller, B.B., Phillippi, J., Péault B., Huard, J. Improved cellular cardiomyoplasty via human myogenic-endothelial cells identification of a progenitor cell population from human skeletal muscle that is superior to committed skeletal myoblasts for cardiac cell therapy. *Journal of the American College of Cardiology*. (2008)

CONCLUSIONS

The experiments performed in this project have provided evidence for the existence of a strong myogenic potential in cells related to the endothelial cell lineage in the normal, human adult skeletal muscle, and therefore suggest a developmental relationship between vascular cells and myogenic cells. We have indeed confirmed the existence of a novel population of cells present between human muscle fibers that co-express the nuclear Pax7 myogenesis-specific transcription factor and surface antigens that typify endothelial cells: VE-cadherin (CD144) and vWF.

The leading conclusion of the present work is that human muscle-derived myoendothelial cells can regenerate skeletal muscle, quantitatively more efficiently than regular myogenic (satellite) cells. The higher regeneration capacity exhibited by myoendothelial cells is due in part to better resistance to oxidative stress, a likely environmental condition within the injured skeletal muscle. These data suggest the existence of a novel hierarchy within human adult skeletal muscle along which vascular endothelial cells give rise to early myogenic stem cells, which in turn replenish the satellite cell population.

These novel myogenic progenitors are amenable to biotechnological processing; these cells can be sorted to homogeneity by flow cytometry from skeletal muscle, then cultured clonally on the long term, with no major loss of developmental potential, in basic medium, where they proliferate rapidly but do not become tumorigenic. Therefore, the transplantation of such autologous endothelium-related progenitors could be envisioned shortly as a therapy of skeletal muscle diseases and injuries.

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14. Zheng, B., Cao, B., Crisan, M., Sun, B., Li, G.H., Logar, A., Yap, S., Pollett, J.B., Drowley, L., Cassino, T., Gharaibeh, B., Deasy, B., Huard, J., Péault, B. Prospective identification of myogenic endothelial cells in human skeletal muscle. *Nature Biotechnology*, 25(9):1025-1034 (2007
15. Crisan, M., Deasy, B., Gavina, M., Zheng, B., Huard, J., Lazzari, L., Péault, B. Purification and long-term culture of multipotent progenitor cells affiliated with the walls of human blood vessels: myoendothelial cells and pericytes. *Methods In Cell Biology*, 86:295-309. (2008)
16. Crisan, M., Zheng, B., Sun, B., Yap, S., Logar, A., Huard, J., Giacobino, J.P., Casteilla, L., Péault, B. Purification and culture of human blood vessel-associated progenitor cells. Current Methods in Stem Cell Biology. In Press.
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Project # 4 Final Report **
Cell therapy for muscle regeneration advances via interdisciplinary-driven regenerative medicine (iDREAM)
(Bridget Deasy)

****Note that this subproject was completed last February 2009. Below is a reiteration of last year's final report**

Key Research Accomplishments

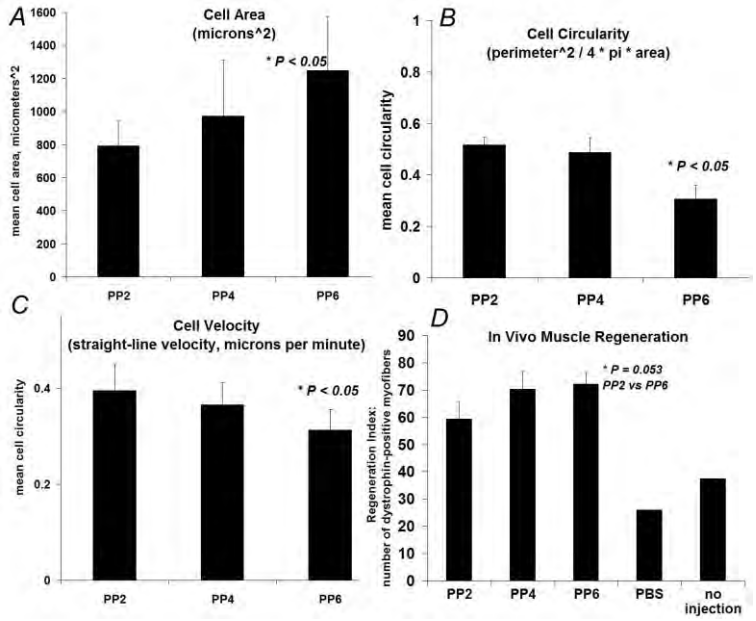
1. **Technical Objective #1:** Use preplate isolation technique and a bioinformatic cell culture system to screen adult human stem cell candidates for their muscle regeneration potential.
2. **Technical Objective #2:** Test culture conditions for extensive and long-term expansion of human myogenic cells to preserve the cells' phenotype and regeneration efficiency.

We have completed these aims. First we have identified that the most potent human muscle stem cell candidate can be isolated from slowly adhering fractions of the cell suspension which derives from the muscle biopsy. To identify these cells we first examined the cells via live cell imaging, and second we examined the cells in an in vivo model of muscular dystrophy.

Through a collaboration with Cook MyoSite Inc (Pittsburgh, PA), we received samples of human muscle cells. These cells were isolated based on their variable adherence, using a method similar to the preplate technique which was performed with mouse myogenic cells. It has been previously shown that the preplate isolation technique can be used to isolate stem cells from the skeletal muscle of mouse [32] and rat [75], while direct defined FACS-sorting has been performed using tissue from mouse [32], rat [75] and human sources[18]. Usually, myogenic precursors with characteristics of myoblasts or satellite cells are found within the early preplates (PP1–3), whereas cells isolated from the later preplates (PP5-6) have stem cell characteristics[1]. We term the cells PP2, PP4 and PP6-- PP2 adhere prior to PP4 which adhere prior to PP6.

These results provide examples that illustrate a relation between in vitro and in vivo characteristics. Human MDCs were analyzed for the presence of the cell surface cluster of differentiation markers CD34, CD56, CD144, and CD146 by flow cytometry. For live cell imaging analysis 3 separate hMDCs preplate populations were examined – PP2, PP4, and PP6. By the preplate method, PP2 corresponds to myoblasts and PP6 corresponds to the muscle-derived stem cell candidate[1,77,79]. The cells were imaged using the automated cell imaging system[81]. Three separate visible image sequences (20x) were captured at 10 minute intervals for each well over a culture time of 3 days. Image sequences were imported to ImageJ for user interactive analysis. Measurements included 17 parameters including area, centroid, center of mass, perimeter, circularity, skewness, and best fit ellipse. Sequences were divided into 18 hour increments with 10 separate cells outlined and measured over 30 minutes at each time point. Results from ImageJ measurement were imported into a predesigned worksheet for calculation and analysis. Cell motility was described as centroid velocity, dividing the change in centroid position by the scan time interval. Single cell measurements were averaged over each time point and image sequence yielding a

Figure 1



morphological description of each well for the culture period. The mean area for PP2 (792±151 um^2) was significantly smaller (P=0.022, type-2, 2-tailed) than PP6 (1248±327 um^2, **Fig 1A**). Furthermore, PP2 velocity (0.395±0.056 um/min) was significantly greater (P=0.031) than PP6 (0.312±0.044 um/min, **Fig 1B**). Finally, examination of circularity (a measure of cell roundness) showed PP2 (0.517±0.028) and PP4 (0.487±0.058) were significantly different from PP6 (0.307±0.052, P<0.01, **Fig 1C**). Flow cytometry showed all PPs were negative for CD34 & CD144, with decreasing expression for CD146 & CD56 over time in culture. In sum, the results show that PP2 cells are significantly smaller and more motile with greater circularity than PP6, two populations previously resolved only by adherence rates. The LCI approach shows the ability to resolve differences in cell populations based on a number of behavioral measures only a few of which are presented here. Moreover subsequent in vivo experiments showed that this in vitro difference correlates with an in vivo difference in performance (**Fig 1D**).

We have also expanded these cells under various cell culture conditions and we confirm that the phenotype of the cells is altered through standard culture conditions (**Fig 2**). We found that cell lose expression of the myogenic marker CD56 and the progenitor marker CD146 as they are expanded (**Fig 3**) as the are expanded.

It is worth noting that whereas we have expanded mouse MDSC populations to beyond 200 population doublings (PDs), our results to date show that human MDSC populations will reach senescence much sooner than mouse MDSCs when grown in the same growth medium of 20% serum Dulbecco's Modified Eagle Medium (DMEM). **Figure 2A** depicts these differences in the proliferation kinetics of human and mouse MDSCs (in 20% DMEM, 10^9 mouse MDSCs vs. 10^{12} human MDSCs, at 7 weeks, n=1). These results underscore the need to more extensively study growth factor-expansion methods for human stem cells. Using commercially available, chemically defined media, we were able to stimulate human muscle–derived stem cell expansion in the *in vitro* expansion assay (**Figure 2**). We have observed that other human muscle cells-myoendothelial cells-

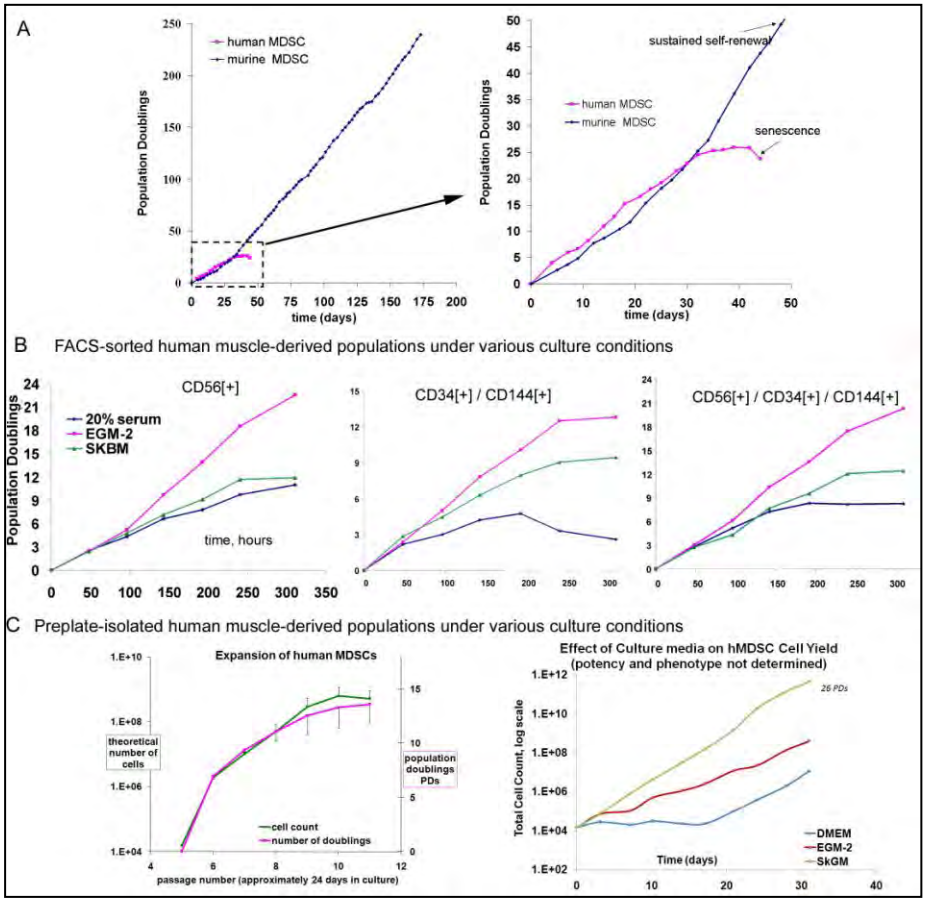


Figure 2. Human MDSC expansion. (A) hMDSC have slower proliferation rates as compared to mouse MDSC *Human myogenic cells were cultured using the same techniques used for mouse MDSCs; yet, a striking difference in their expansion potential was observed.* ***(B)*** *Expansion of FACS-isolated human myogenic progenitors was examined using various culture media (20% serum, EGM2 and SKGM). Medium supplemented with endothelial growth factor (EGM2) stimulated the greatest amount of expansion for all hMDCs. The results of panel (B) were published in Zheng et al 2007 Nat Biotech.* ***(C)*** *While hMDSC show promise for clinical treatment of urinary incontinence much work needs to be performed to expand these cells to higher cell doses to reach a much larger set of tissues to be effective for muscular dystrophies.*

have exhibited increased expansion when cultured in defined media such as endothelial growth medium 2 (EGM2, Clonetics) or skeletal basal medium (SKBM, Clonetics) ([56], **Figure 2B**). Expansion of FACS-isolated human myogenic progenitors was examined using 3 different culture media (20% serum, EGM2 and SKGM [56]). Three purified populations were examined – CD56[+] cells, CD34[+]CD144[+] cells and CD56[+] CD34[+] CD144[+] cells. All three purified human populations demonstrated the highest degree of expansion with EGM-2 media **(Figure 2B).**

The results shown in **Figure 2B** were recently included in a report by Zheng et al, in which the authors suggest that the MDSCs may have their developmental origins as myoendothelial cells[56,57]. Subsequently we performed another examination of the effect of 3 different culture media on the preplate derived human MDSC populations. In this case, we observed the greatest expansion potential with SKGM (**Figure 2**), and not EGM2 which favored human myoendothelial cells sorted by FACS. While this may suggest that the human myoendothelial cells and the human preplate MDSCs are unique populations, those experiments have not been performed side-by-side, and it is not our goal to compare these populations. Rather, these findings show that optimization of cell expansion is cell-line specific and the human preplate cells can be expanded to high numbers using SKGM as compared to EGM2 (**Figure 2**). We examined phenotype during the course of cell expansion. All 3 preplate fractions were initially negative for CD34 and CD144 (data not shown). The PP2, PP4, and PP6 fractions expressed significantly different amounts of the myogenic marker desmin (**Figure 3A**) with the PP2 (fast adhering) fraction containing the highest percentage of positive cells ($p < 0.05$ as compared to the slow adhering or PP6 fraction). We also observed variable levels of CD56 and CD146, and these markers decreased with cell passage. **Figure 3B** shows the changes in CD56 which occur as cells are cultured. The fast-adhering (PP2) fraction, and moderately adhering (PP4) fraction show a decrease in CD56 expression through the course of 3 cell passages (passage 7 through passage 9, **Figure 3B**). The slow (PP6) fraction maintains a low level of CD56 through the course of these passages. We have also examined the PP6 / hMDSCs fraction for expression of CD90, CD105 and CD73, which are expressed by mesenchymal stem cells. The populations are >90% positive for these markers (flow cytometry and PCR results, data not shown). These results and others suggest that hMDSCs may be obtained from the PP6 fraction which appears to be distinct from PP2.

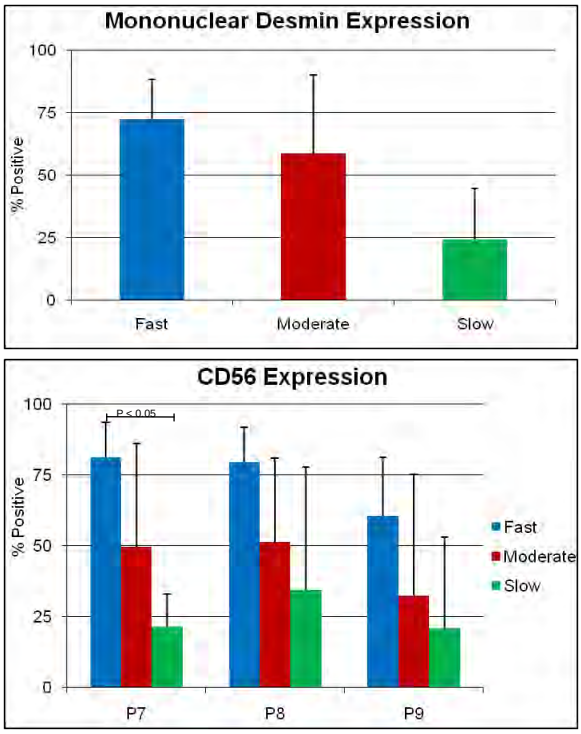


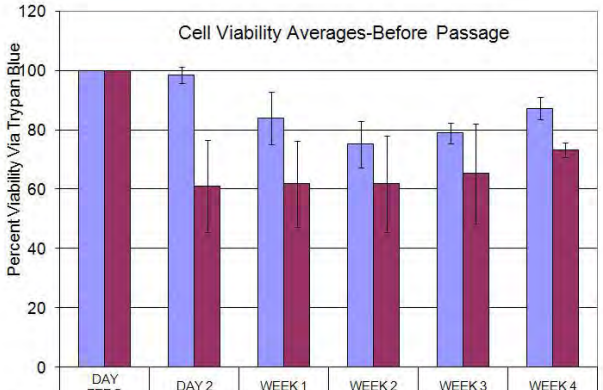
Figure 3. *Human preplate populations. PP2=Fast adhering fraction, PP4=moderate rate of adherence, PP6=slow adherence rate during the preplate isolation process. These populations differ in their expression of* ***(A)*** *desmin (via immunocytochemistry) and* ***(B)*** *CD56 (via flow cytometry). We also illustrate in (B) that the expression of this marker changes with cell expansion (passage 7-passage 9 are shown).*

Supplemental Objectives

Technical Objective #1: Use a bioinformatic LCI culture system to test the effect of temperature on the processing of MDSC from skeletal muscle biopsy. Here we will consider the various temperature cycles that the biopsy may undergo prior to stem cell isolation. We will examine differences in percent yield based on the length of time that a biopsy is at 4C storage. We will examine the phenotypes of MDSCs from various isolations using the LCI tools.

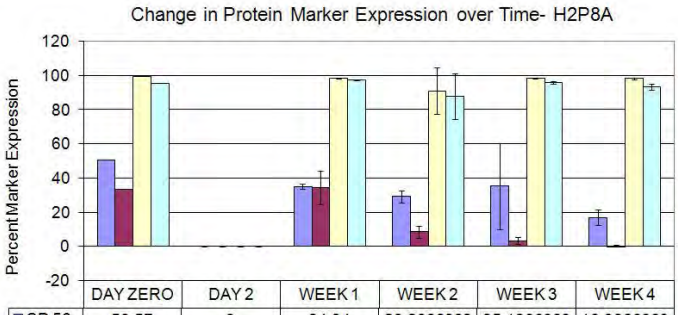
Progress: The instrumentation for these studies is now in place and we have initiated studies of the effects of temperature on stem cell viability. The delivery of the imaging system for these experiments was delivered on Jan 14, 2009 and calibration was complete on Jan 30, 2009. Therefore, it was necessary for us to request a no-cost extension on these objectives. We have performed initial experiments to examine cell viability at lower temperatures and to date we confirmed viability and cell proliferation, however, the complete analysis of the effect of temperature is still ongoing.

A



	DAY ZERO	DAY 2	WEEK 1	WEEK 2	WEEK 3	WEEK 4
H2P8 A- 10% DMSO	100	98.41269841	84.01292335	75.13597514	78.93939394	87.24747475
H2P8 B- 20% DMSO	100	61.02693603	61.81318681	61.80555556	65.27777778	73.21428571

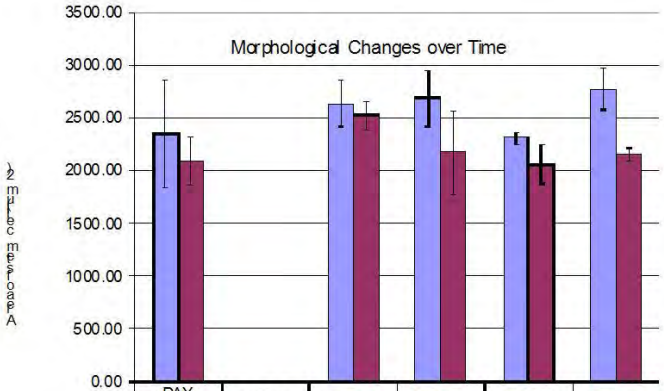
B



	DAY ZERO	DAY 2	WEEK 1	WEEK 2	WEEK 3	WEEK 4
CD 56	50.57	0	34.94	29.2033333	35.1866666	16.9066666
CD 146	33.27	0	34.35	8.46666666	3.31	0.30333333
CD 73	99.36	0	98.38	90.95	98.3533333	98.16
CD 105	95.57	0	97.31	87.86	95.78	93.3266666

Technical Objective #2: To test the effect of various cyroprotectant agents and procedures on the stem cell phenotype of human MDSC. In this objective, we will obtain unique comprehensive phenotype profiles of muscle-derived stem cell populations after various preservation protocols. We will also examine various thawing procedures and test the percent cell recovery after thawing. Most important we will test the quality of the stem cells to perform in multilineage differentiation assays. We will use the optimal human MDSC phenotype (identified in DOD 2005-2007) to identify which processing procedures are optimized for maintaining stem cell quality and quantity.

C



	DAY ZERO	DAY 2	WEEK 1	WEEK 2	WEEK 3	WEEK 4
H2P8 A- 10% DMSO	2357.32	0.00	2640.07	2685.01	2314.12	2775.48
H2P8 B- 20% DMSO	2090.50	0.00	2530.19	2174.56	2061.97	2155.19

Figure 4. We have already examined the effect of DMSO and glycerol on the viability and stability of another cell type which is being using in human clinical trials for urinary incontinence (muscle derived cells, MDCs, Cook Myosyte). In this collaborative study, we observe the best post-thaw recovery after freezing with 10% DMSO and compared to 20% DMSO (glycerol not shown)

Progress: We have identified the optimal MDSC population as presented above and we have examined several cryoprotectants agents to examine the effect of freezing on the phenotype and cell viability. Human muscle stem cells which may be used in a clinical setting for cell therapeutics will need to be harvested, processed in a GMP facility, and cryopreserved prior to use. Each of these steps will expose the cells to reagents which may damage the cells or induce phenotypic changes. In addition, each step may occur at a different temperature, and the stem cells are therefore exposed to temperature cycles which are potentially damaging.

We currently collaborate with a local biotech company CookMyosite which studies human muscle-derived cells for use in urinary incontinence. In fact this company is currently conducting clinical trials transplanting these cells to the urethra of patients with urinary incontinence. In the Deasy lab, we have studied the effect of cryopreservation on these cells. In these studies, human muscle stem cells were added to a solution of fetal bovine serum (FBS) and a cryoprotectant (DMSO or glycerol) at 100,000 cells per cryopreservation vial. We examined 6 timepoints (day 0, day 2, week 1-4), done in triplicate. At the given timepoints, cells are slow frozen in the -80°C freezer, then quick thawed in the room temperature waterbath. For each time point , cells are thawed from cryopreservation vials, counted for viability via Trypan Blue, put in culture for 24 hours, imaged in T-25 plates, then passaged, counted again for viability via Trypan Blue (**Fig.4a**) . Remaining cells are flowed through the flow cytometer to determine percent marker expression (**Fig.4b**). Images of cells in cultured in T-25 analyzed in Image J for morphological changes (**Fig.4c).**

Under the conditions studied here, we observe a moderate about of cell death which could be improved by using higher concentrations of DMSO. Slow freezing stem cells kills around 50% cells from day 0 to week 4. We observe that10% DMSO is a better concentration than 20%- provides increased cell viability. Thawed muscle cells lost their expression of protein markers CD56 and CD146. H2P9A exhibited a 67% decrease in CD56; 99% decrease in CD146. H2P9B exhibited a 68% decrease in CD56; 97% decrease in CD146. H2P8A cell area increased by 18%; H2P8B cell area increased by 3%. There also was a significant difference in the number of cells remaining at week 4 compared to day 0, and there was significant difference in the percent marker expression of CD56 and CD146 comparing day 0 to week 4 ($p < 0.05$). There is no significant difference in area between day 0 data and week 4 for both samples.

In this Phase I project, we will focus on the effect of cryopreservation on human umbilical cord derived cells. Currently, dimethyl sulfoxide, DMSO, is the cryopreservation reagent utilized in the preservation of these cells. The cells are normally frozen in a solution of 10% DMSO, and stored at -80 C or -196 C, for days, weeks, or months. For clinical applications, human MDSCs have been shipped in a frozen state from the processing facility to the clinic(*56, 116*). It is key that these cells retain their stem cell phenotype after thawing.

Recommended changes: In the original protocol, we proposed to examine the effect of temperature fluctuations of cell phenotype. We propose to continue with these aims. We also have no recommended changes to the study of cryopreservation of the human muscle-derived cells.

Publications which have resulted from this work:

1. Chirieleison SM, Schugar RC, Deasy BM. 2008. Current Progress in Cell-mediated Gene Therapy for Muscular Dystrophies. *Research Advances in Gene Therapy*. Global Research Network. Editor: RM Mohan.
2. Schmidt, BT, Feduska, JM, Witt, AM, and BM Deasy. 2008 Robotic cell culture system for stem cell assays. *Industrial Robot*. *35 (2):116-124.*

Abstracts that have resulted from this work :

1. Chirieleison SM , Scelfo CC, Askew Y, Deasy BM. 2008. Development of Morphological Measurement Scheme Using Open Source Software for Live Cell Imaging Approaches to Stem Cell Biology. University of Pittsburgh Science 2008; Pittsburgh, PA, USA. POSTER
2. Chirieleison SM , Scelfo CC, Deasy BM. 2008. Quantitative Analysis of Therapeutic Muscle Cell Populations through Live Automated Cell Imaging. American Society for Cell Biology Annual Meeting; San Francisco, CA, USA. POSTER
3. Chirieleison SM , Scelfo CC, Askew Y, Deasy BM. 2008. Development of Morphological Measurement Scheme Using Open Source Software for Live Cell Imaging Approaches to Stem Cell Biology. Pittsburgh Orthopaedic Journal; Pittsburgh, PA, USA.
4. Scelfo CC, Chirieleison SM , Deasy BM. 2008. Integration and Adaptation of Live Cell Image Analysis. Pittsburgh Orthopaedic Journal; Pittsburgh, PA, USA.

5. Chirieleison SM , JM Feduska, RC Schugar, SL Sanford, J Huard, BM Deasy. 2008. Identifying Populations of Human Muscle Derived Stem Cells to Participate in Skeletal Muscle Regeneration Based on Phenotypic Differences. Pittsburgh Orthopaedic Journal; Pittsburgh, PA, USA.

6. Chirieleison SM , Feduska JM, Schugar RC, Witt AM, Deasy BM. 2008. In Vitro Aging of Human Muscle Stem Cells Due to Culture Expansion. International Society for Stem Cell Research; Philadelphia, PA, USA. POSTER

7. Chirieleison SM , Scelfo, CC, Askew Y, Deasy BM. 2008. Development of Morphological Measurement Scheme Using Open Source Software for Live Cell Imaging Approaches to Stem Cell Biology. International Society for Stem Cell Research, Philadelphia, PA, USA. POSTER

8. Chirieleison SM , Feduska JM, Schugar RC, Sanford SL, Huard J, and Deasy BM. 2008. Identifying Regenerative Populations of Human Muscle-Derived Cells and Tracking Phenotypic Changes in Culture. MidWest Tissue Engineering Consortium. Cincinnati, OH, USA. PODIUM

9. Chirieleison SM , Feduska JM, Schugar RC, Sanford SL, Huard J, Deasy BM. 2008. Identifying Populations of Human Muscle Derived Stem Cells to Participate in Skeletal Muscle Regeneration Based on Phenotypic Differences. Orthopaedic Research Society; San Francisco, CA, USA. POSTER

Project # 5 Final Report**
Inhibiting cell death and promoting muscle growth for congenital muscular dystrophy
(Xiao Xiao)

****Note that this subproject was completed last February 2009. Below is a reiteration of last year's final report**

Introduction

Congenital muscular dystrophy (CMD) is a group of severe forms of muscular dystrophy leading to early death in human patients(1-3, 7). The majority of cases are caused by genetic mutations in the major laminin, laminin containing the α2 chain (formerly named merosin), in the muscle basement membrane(4, 6, 8, 11). The early morbidity/fatality and the lack of effective treatment require urgent search for novel therapeutics. Previously, we utilized mini-agrin, which has been proven to have a therapeutic effect in transgenic MCMD mice, to treat MCMD mice by AAV vector(10). Our preliminary studies showed that over-expression of mini-agrin protein greatly improved general health and muscle morphology in MCMD mice. However, the treated disease mice still developed gradual paralysis and displayed shorter life span than wild type mice. To further improve the current gene therapy paradigm, we have developed complementary gene therapy strategy for laminin alpha2-deficient CMD.

Body

As mentioned earlier, we utilized laminin alpha2 deficient congenital muscular dystrophy (MDC1A), a severe form of muscular dystrophy animal model, as our muscle injure model in this proposal. We evaluated the therapeutic effects of delivering different molecules, such as insulin-like growth factor 1 (IGF-1), anti-apoptotic gene BCL-XL, as well as myostatin propeptide, on muscle and nerve pathogenesis of MDC1A. While we did not observe the significant therapeutic effects by single delivery of those factors on MDC1A mice, we made very interesting discovery during those experiments and wrapped up two manuscripts. Since the first manuscript has been published {Qiao, 2008 #980}, we will mainly concentrate on the second manuscript in this report.

AAV vector transduced white matter of spinal cord

Previously, we have revealed that delivering AAV serotype 8 vectors to neonatal mice can efficiently transduce whole-body skeletal muscle and cardiac muscle. In the present study, we utilized the same AAV serotype and similar delivery route to investigate the ability of AAV vector to infect peripheral nervous system and spinal cord. For the first set of study, we utilized AAV8-CMV-LacZ vector. Two months after vector injection, the mice were sacrificed. The whole spine including bone and adjacent muscle were cut into three pieces and quick-frozen away. After cryo-section, the slides were subjected to LacZ staining, and H& E staining were

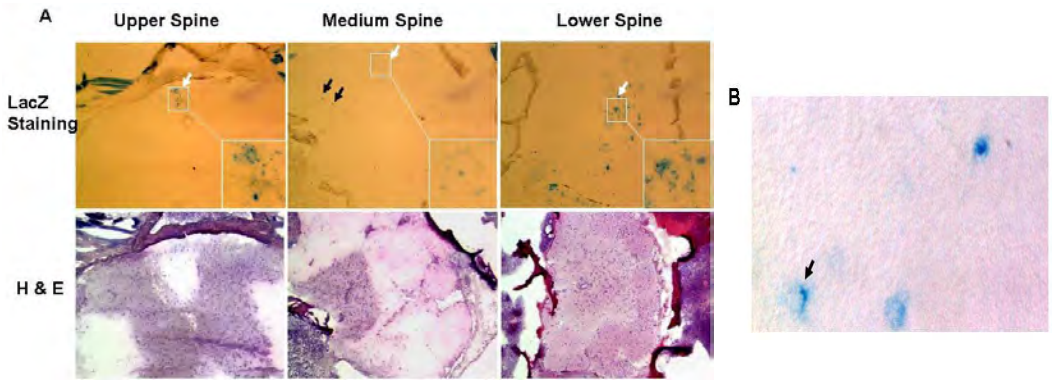


Figure 1. LacZ staining of spinal cord from AAV8-LacZ injected mice. A. LacZ positive cells in white matter. The vector was delivered on 3-day-old neonatal mice, and the mice were sacrificed at 2-month-old age. Notably, most of the lacZ positive spots were in white matter (white arrow), and very few LacZ positive cells were in grey matter (black arrow). Insets were indicated enlarged area. The consecutive sections of H&E staining were used to display the structure of spinal cord. It was also apparent that there were more LacZ positive cells in lower spine, followed by medium spine, and then upper spine. B. Few LacZ positive cells in grey matter. Button-like structure (black arrow) in grey matter indicated small neuron and axon communication.

performed on the consecutive sections to display the histology. As shown in figure 1A, most LacZ positive cells were located in white matter in the spinal cord of AAV8-LacZ delivered mice (white arrow and inset area). Occasionally, we could see few LacZ positive cells in grey matter (black arrow and figure 1B). Notably, there were more LacZ positive cells in the lower spine and less LacZ positive cels in the higher spine potion. Large magnification of few LacZ positive cells in grey matter of ventral horns showed button-like structures in close proximity and overlapping neuronal cells (Fig 1B). AAV8 vector effectively transduced dorsal root ganglion and peripheral nerves

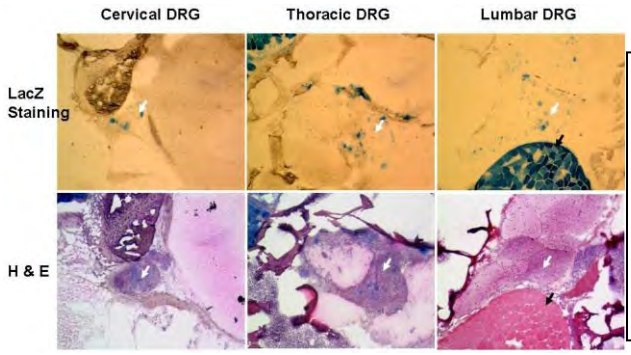


Figure 2. LacZ staining indicated AAV8-LacZ vector efficiently transduced dorsal root ganglion (DRG). White arrow indicated DRG, and the consecutive section of H&E staining were utilized to display the DRG structure. Black arrow pointed to adjacent LacZ positive muscle cells

In addition to the white matter, we noticed AAV8 vector could efficiently infect dorsal root ganglion (DRG) cells which were positioned outside of the spinal cord (Fig2 and Fig 3, white arrow). Across the whole spine, all the DRG neuronal cells were effectively transduced by AAV vector (Fig.2).

In the second set of our study, we wished to use a different report gene, such as GFP gene, to further prove our finding. Similar to LacZ vector, AAV8-D(+)-CMV-GFP vector was delivered into neonatal mice. Two months after vector injection, the mice were sacrificed. To reduce the background staining of spinal cord, we did perfusion to remove circulating blood and fixed with 4% paraformaldehyde (under anesthesia) when the mice were sacrificed. Figure 3 displayed middle portion of the spine, most of the GFP positive cells were located in while matter of spinal cord and DRG (white arrow).

Except for white matter of spinal cord and DRG, we also observed LacZ and GFP positive cells in peripheral nerve fibers (Fig 4).

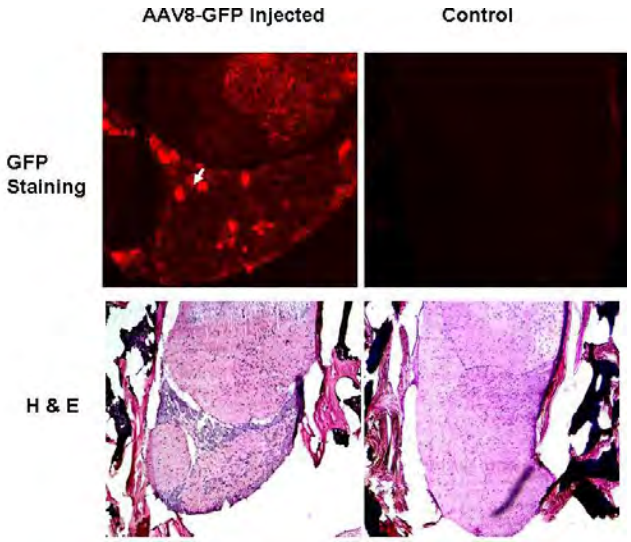


Figure 3. Immunofluorescent staining of GFP in spinal cord and dorsal root ganglion from AAV8-GFP delivered mice.

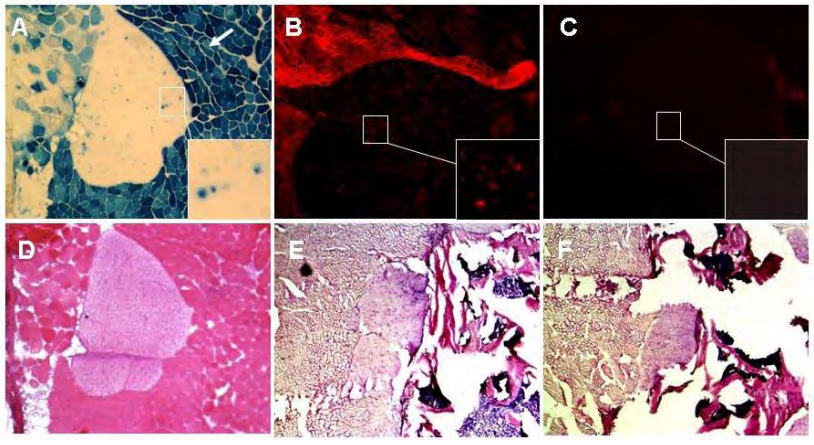


Figure 4. AAV vector efficiently transduced peripheral nerve roots. A was from AAV8-LacZ injected mice, and B was from AAV8-GFP injected mice, C was from mock injected animal but stained with GFP. D, E, F were consecutive section of H& E staining. Insects were indicated enlarged area of peripheral nerves. The LacZ positive cells surrounding peripheral nerves in picture A (white arrow) were muscle cells.

Key Research Accomplishments:

1. We have delivered myostatin propeptide gene into laminin alpha2-deficient CMD mice and the therapeutic effect was evaluated. Unlike mdx mice for which myostatin propeptide delivery can ameliorate its muscle pathology, the delivery of propeptide had a severe side effect on laminin alpha2-deficient CMD mice. The injected mice died even early than untreated controls. This phenomenon is consistent with previous discovery(5).
2. We have delivered AAV-IGF1 vector into laminin alpha2-deficient CMD mice and did not observe significant therapeutic effect.
3. We have delivered AAV-BCL-XL into laminin alpha2-deficient CMD mice and did not observe significant therapeutic effect.

Reportable Outcomes:

Our manuscript entitled –Myostatin propeptide gene delivery by AAV8 vectors enhances muscle growth and ameliorates dystrophic phenotypes in *mdx* mice" has been published in Human Gene Therapy 2008 Mar; 19(3): 241-54(9).

Our second manuscript titled –Efficient gene transfer to dorsal root ganglion and peripheral nerves via systemic delivery of AAV vectors" is in preparation and will be submitted soon.

Conclusions:

Single AAV-IGF1, AAV-Bcl-XL, or AAV-MPRO gene delivery can not offer therapeutic benefit for laminin alpha 2 deficient dy^w/dy^w mice.

AAV can efficiently transduce peripheral nervous system and dorsal root ganglion cells.

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Project # 6 Final Report**
Treatment for Muscle Wasting
(Paula Clemens)

****Note that this subproject was completed last February 2009. Below is a reiteration of last year's final report**

Introduction:

The ability to promote muscle regeneration in the setting of focal or generalized muscle loss could confer significant clinical benefit in the setting of focal neuropathic or other processes that cause muscle atrophy or chronic illnesses that cause cachexia. We hypothesize that gene transfer strategies can promote muscle regeneration toward a goal of improving muscle bulk and strength in the setting of injuries or diseases that cause muscle atrophy.

Extensive evidence has shown that higher levels of some pro-inflammatory cytokines contribute to the development of cachexia. For example, serum tumor necrosis factor α (TNFα) and IL-1α levels are markedly increased in patients with rheumatoid arthritis and cancer. Other cytokines, such as IL-6, IL-1β and proteolysis-inducing factor (PIF) have been reported to contribute to the development of muscle wasting.

Elegant studies show that TNFα binds to its receptor on skeletal muscle resulting in the activation of nuclear factor κB (NF-κB) mediated by phosphorylation and degradation of the NF-κB inhibitory protein, IκBα. Downstream effects of pathological NF-κB activation in skeletal muscle include the inhibition of new muscle formation and the degeneration of existing muscle. *In vitro* studies support the potential that the IκBα superrepressor (IκBSR), an IκBα genetically engineered to prevent its phosphorylation, can prevent the activation of NF-κB in skeletal muscle and could ameliorate or prevent muscle wasting. Our preliminary studies demonstrate the novel determination of inhibition of activation of NF-κB by cellular FLIP (cFLIP).

Our hypothesis is that the inhibition of the downstream pathways of TNFα causing muscle atrophy and failure of muscle regeneration should be effected intracellularly in muscle fibers. A gene therapy strategy is ideal for the purpose of achieving an ongoing effect that is cell-type restricted. We anticipate that inhibition of NF-κB activation restricted to muscle fibers will provide a therapeutic effect on muscle while avoiding potential toxic effects of inhibiting NF-κB activation in other tissues.

Body:

To characterize an *in vitro* model of cancer-induced muscle wasting in primary muscle cells and in stable muscle cell lines expressing IκBSR or cFLIP.

We developed a new *in vitro* cell culture assay to study the effects of cancer cell cytokines on muscle cell differentiation and use this assay to test novel gene transfer approaches for the treatment of cancer cachexia. Exposure to conditioned media from selected human cancer cell lines resulted in failure of muscle cell differentiation. A known intracellular mechanism of NF-κB activation as a cause of cancer cachexia was recapitulated in this *in vitro* system. We observed a direct correlation between NF-κB activation and inhibition of myogenic differentiation in the *in vitro* assay. Exposure to inflammatory cytokines and to conditioned media from human cancer cells each resulted in NF-κB activation within primary muscle cells. Failure of myogenic differentiation and the associated activation of NF-κB were prevented by stable expression of either IKBSR or cFLIP, but not by Bcl-xL. These findings were published. As described below, we used the PC-3 cell line to generate a new *in vivo* cancer cachexia model in mice.

Taken together, this study provides an *in vitro* assay that demonstrates secretion of cachexia-inducing factors by certain cancer cell lines that result in the inhibition of myogenic differentiation by activation of NF-κB. Over-

expression of cFLIP in muscle cells inhibits both NF-κB-mediated and apoptotic pathways, thereby preventing tumor media-induced inhibition of myogenic differentiation and cytotoxicity. These findings point toward the potential to design novel molecular therapeutics for the treatment of cancer-induced muscle wasting.

To clone, rescue, and purify adeno-associated viral (AAV) vectors carrying IκBSR or cFLIP.
We are generating vectors with either ubiquitous or muscle-specific promoter control of expression. AAV serotype 8 vectors provide high levels of muscle transduction and the ability to transduce muscle by systemic delivery. Where possible, we are using self-complementary vectors that result in the highest possible levels of transgene expression.
At our last report, we had rescued the following vectors as high-titer, purified AAV8 vectors: Conventional single strand AAV8 vectors were rescued with the following expression cassettes: CMV-GFP and CMV-cFLIP. Self-complimentary (double strand) AAV8 vectors were rescued with the following expression cassettes: CMV-IκBSR and MCK-GFP. The double strand MCK-GFP vector has been tested by direct intramuscular injection showing high levels of transgene expression.

Since the last report, we rescued the MCK-IκBSR double strand vector as AAV8. The MCK-cFLIP double strand vector plasmid was difficult to clone, but we finally achieved it. We are currently rescuing that plasmid as AAV8. We observed widespread skeletal muscle expression from the MCK-GFP AAV8 vector after systemic delivery. We also observed skeletal muscle expression after intramuscular and systemic delivery of the MCK-IκBSR AAV8 vector.

Characterization of a new PC-3 cell cancer cachexia model in mice.
We have refined our model of muscle cachexia in mice in order to test molecular therapeutics *in vivo*. We generated a novel model of cancer cachexia induced by PC-3 cells and compared it to an established model induced by MCA-26 cells. We reported initial findings on the *in vivo* PC-3 cell cancer cachexia model in our last report. We have now completed those studies and prepared a manuscript for submission. We showed that tibialis anterior and quadriceps muscles had increased activation of NF-κB in the PC-3 cell model of cancer cachexia, as predicted from our *in vitro* studies exposing muscle cells in culture to supernatant from PC-3 cells. Interestingly, the gastrocnemius, which did not show an increase in activated NF-κB, instead showed increased levels of phosphorylated eukaryotic initiation factor 2 alpha (p-eIF2-α) indicating decreased protein synthesis contributing to the cachexia phenotype. This raises the interesting possibility that increased protein degradation or decreased protein synthesis may contribute to cachexia to different degrees in different muscle groups.

To apply AAV8 vector strategies to ameliorate cancer cachexia.
Five-week old CD2F1 mice received intramuscular administration of either AAV8-MCK-dsIκBSR or AAV8-CMV-dsIκBSR or AAV8-CMV-ss cFLIP vector 1 week prior to MCA-26 tumor inoculation. Body weight and tumor growth were monitored every other day. All mice developed tumors and cachexia beginning at about 12 days after tumor inoculation (Figure 1). Three weeks tumor after innoculation the mice were sacrificed and hind-limb muscles were dissected, weighed, and snap-frozen for analysis. The tibialis anterior muscle injected with AAV8-MCK-dsIκBSR showed a significant increase in weight compared to tumor-bearing control (Figure 2). Tibialis anterior muscle injected with each of the 3 therapeutic AAV8 vectors showed a significant increase in muscle fiber diameters compared to tumor-bearing control (Figure 3).

Our results suggest that vectors expressing proteins with the intended effect of decreasing NF-κB activation can ameliorate cancer cachexia *in vivo*. Future studies are needed to test vector-mediated gene delivery of IκBSR and cFLIP by systemic delivery in cancer cachexia models.

Figure 1A

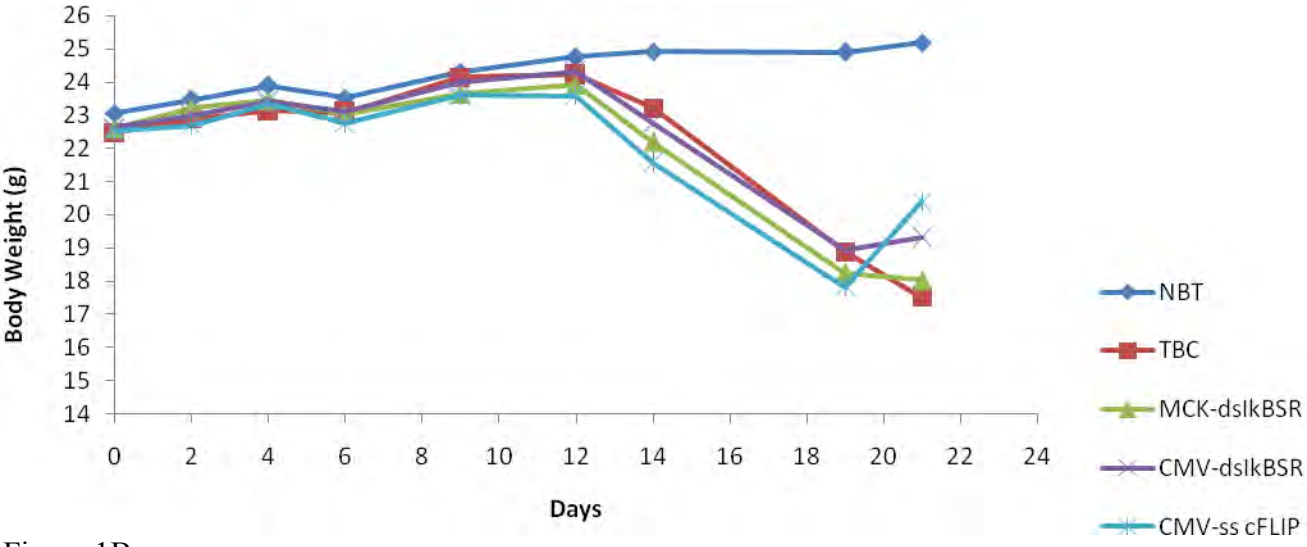


Figure 1B

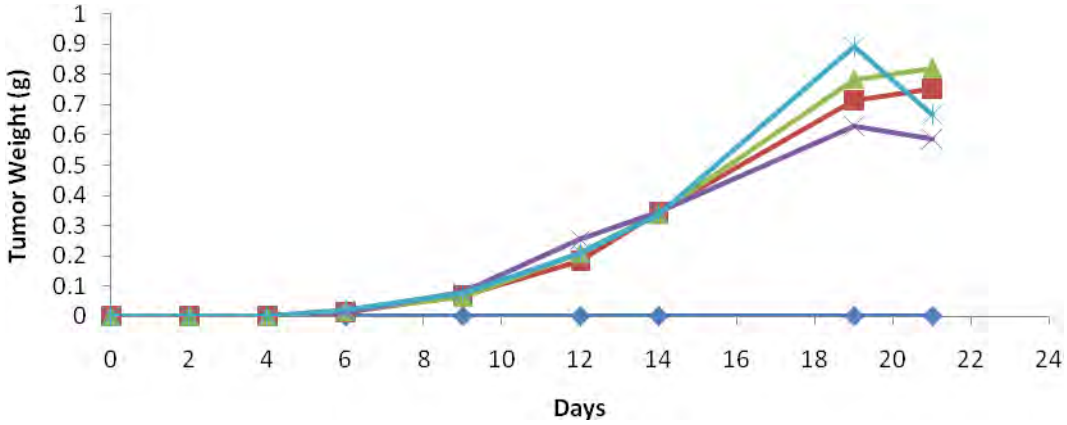


Figure 1. Effect of intramuscular administration of AAV8 carrying MCK-I□BSR or CMV-I□BSR or CMV-cFLIP on body and tumor weight of MCA-26 tumor-bearing in comparison with non-tumor-bearing control (NTB)

CD2F1 mice were intramusculary injected with AAV8 vector carrying MCK-I□BSR or CMV-I□BSR or CMV-cFLIP on tibialis anterior and quadriceps muscle ($3.4x10^{10}$ v.p./muscle) and one week after the mice received MCA-26 tumor inoculation (10^6 cells/mouse) (day 0). (A) Body weight change of mice bearing MCA-26 tumor with or without AAV8 treatment as compared to NTB. (B) Tumor growth rate in MCA-26 tumor-bearing mice with or without AAV8 treatment. The number of mice in each group n=5.

Figure 2

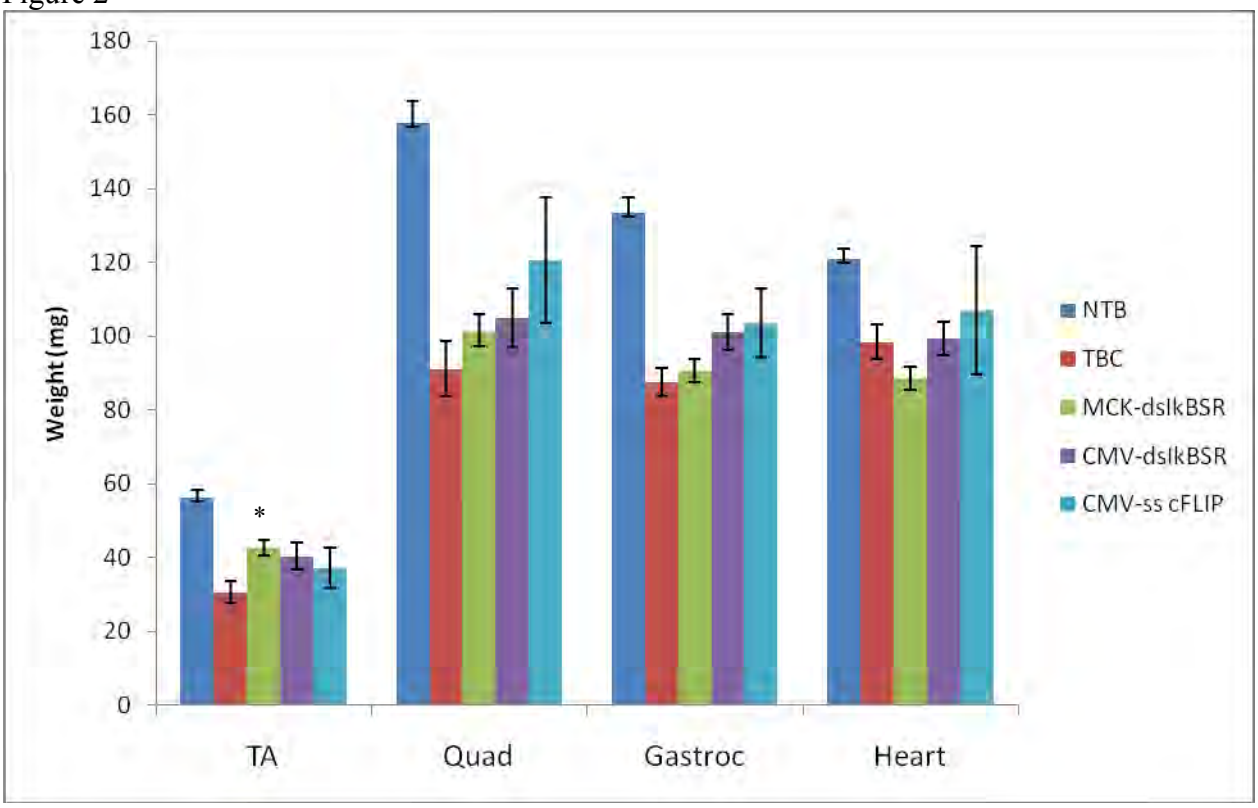


Figure 2. Treatment with AAV8 carrying MCK-I□BSR or CMV-I□BSR or CMV-cFLIP results in improved muscle weight in mice bearing MCA-26 tumor

Mice were sacrificed and hind-limb muscles (TA = tibialis anterior, Quad = quadriceps, Gastroc = gastrocnemius) and heart were collected. The average weight of each individual muscle of NTB and MCA-26 tumor-bearing mice with or without AAV8 treatment are shown. Differences from tumor-bearing without AAV8 treatment (TBC = tumor-bearing control) are shown * = $p < 0.05$.

Figure 3A

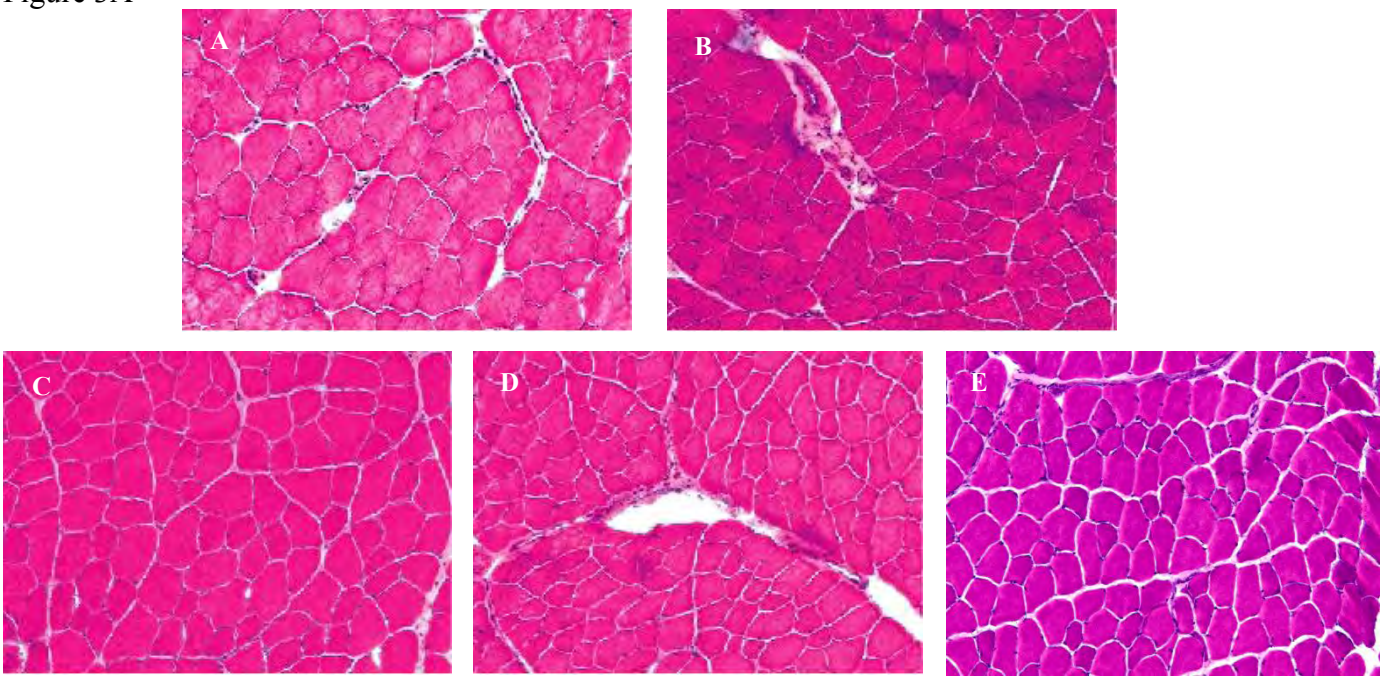


Figure 3B

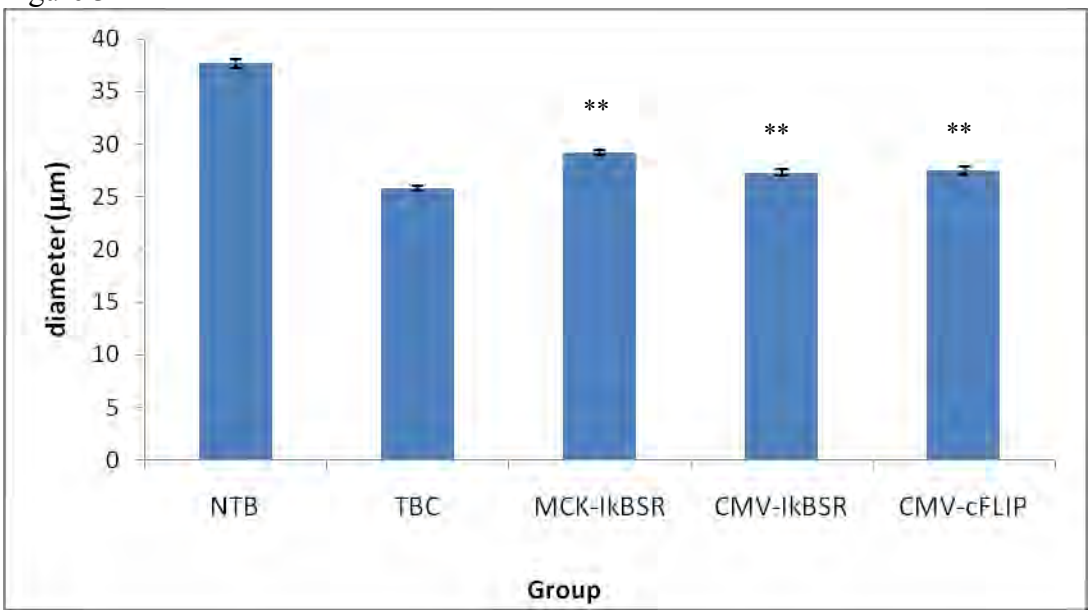


Figure 3. Treatment with AAV8 carrying MCK-IκBSR or CMV-IκBSR or CMV-cFLIP results in increased muscle fiber diameters in mice bearing MCA-26 tumor
Representative H&E staining of TA muscle section is shown in (A); (a) non-tumor-bearing control, (b) MCA-26 tumor-bearing control, (c) AAV8-MCK-IkBSR injected MCA-26 cachexia, (d) AAV8-CMV-IkBSR injected MCA-26 cachexia, (e) AAV8-CMV-cFLIP injected MCA-26 cachexia. The average muscle fiber diameter of each group is shown in (B). Differences from TBC (tumor-bearing control) are shown ** = $p < 0.01$.

Key Research Accomplishments:
- Development of an *in vitro* assay of muscle wasting induced by cancer cytokines
- Demonstration of importance of NF-κB activation as a molecular mechanism for cancer-induced muscle wasting in the *in vitro* assay
- Use of the *in vitro* assay to identify cFLIP as a novel potential therapeutic agent for the treatment of cancer-induced muscle wasting
- Generation of single strand and double strand AAV8 vectors
    - Single strand AAV8 vectors
        - CMV-GFP
        - CMV-c-FLIP
    - Double strand AAV8 vectors
        - MCK-GFP
        - CMV-IκBSR
        - MCK-IκBSR
    - Cloned as plasmid
        - AAV-MCK-D(+)-c-FLIP
- Refinement of 2 cancer cachexia models in mice
- Testing of AAV8 vectors carrying IκBSR and cFLIP for amelioration of cancer cachexia in an *in vivo* murine model

Reportable Outcomes:
Jiang Z, Clemens PR. Cellular caspase-8-like inhibitory protein (cFLIP) prevents inhibition of muscle cell differentiation induced by cancer cells. FASEB J 2006 Dec;20:E1979-E1989.

Sae-Chew P. Clemens PR. A Novel Muscle-Wasting Mouse Model Induced By Human Prostate Cancer Cells: Comparison with a Well Established Model Induced By Murine Colon Adenocarcinoma Cells. Prepared for submission (attached).

Conclusions:
Our studies to date identify an *in vitro* assay that allows us to test molecular therapies that have the potential to treat muscle wasting induced by cancer. We anticipate that these results can be generalized to the treatment of other genetic and acquired causes of muscle wasting. We produced multiple AAV8 vectors with expression cassettes designed to inhibit activation of NF-κB and ameliorate cancer cachexia. We applied several newly developed AAV8 vectors for treatment of cancer cachexia in a murine model.

Vector Core Final Report
(Bing Wang)

Progress made to date (3-1-06 to 12-31-11):

Vector Core of the DOD project (W81XWH-06-1-0406)
Sub-title: The structure, production and purification of adeno-associated viral, adenoviral and retroviral vectors"

Introduction:

The Molecular Therapy Laboratory (MTL) in the Department of Orthopaedic Surgery continued to focus on the construction and expansion of the viral vectors utilized in the latter projects for use in gene transfer and stem cell technologies for treating musculoskeletal diseases, mainly by using recombinant adeno-associated viral (rAAV), retro-viral and lenti-viral vectors which represent the most promising approaches to aid in the repair and regeneration of muscle, bone, ligament, tendon, and joint capsules. In the meanwhile, MTL also serves as a Vector Core of a DOD project (W81XWH-06-1-0406, PI: Dr. Johnny Huard) for the design, construction and production of a lots of viral vectors which are used to gene transfer and genetic engineering of stem cells in the whole project.

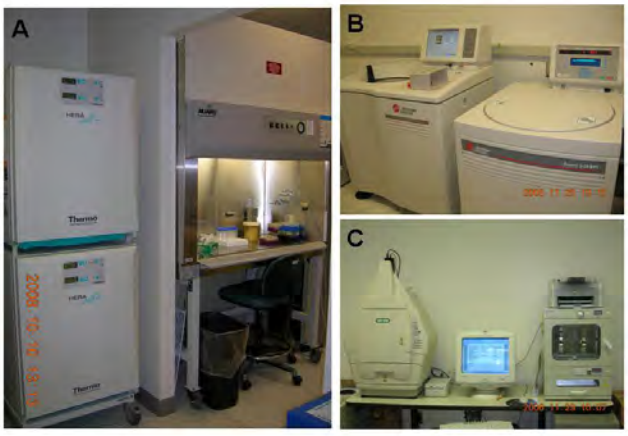


Figure 1. Major Equipment

The Vector Core is located in the Biomedical Science Tower, room E1603. Total dedicated Vector Core space is approximately 500 square feet for molecular biology and viral vector production. Inventory of major equipment includes one tissue culture hood and one temperature-controlled CO2 incubator for cell culture and viral vectors production (**Figure 1 A**). In addition, Vector Core is also equipped with one Beckman TM L-100 XP Ultracentrifuge, one Beckman Avanti TM J-20 XP high-speed centrifuge for the purification of plasmid DNA and viral vectors (**Figure 1B**). Moreover, one Beckman refrigerated table-top centrifuge, two microfuges, one refrigerator, one BioRad PCR machine, one UV spectrophotometer, and one BioRad gel documentation camera (**Figure 1C**), and two BioRad electric gel transfer apparatuses will be used for molecular analysis and vectors construction. Also, one Nikon inverted fluorescent microscope and -30ºC and -80ºC freezers, and one liquid nitrogen tank are established for evaluating the expression of report genes, storages of plasmid DNA, viruses and cell clones.

Body

Development and production of AAV vectors

The Vector Core is actively engaged in multiple applications of rAAV vectors for gene therapy, including different serotypes of AAV vectors, both single and self-complementary AAV vectors, inducible vector system. Moreover, we developed the different promoters in AAV vectors and gene-silencing AAV vectors. As a vector core, we collaborated with many laboratories internal and external the University of Pittsburgh. Our goal is to develop very useful viral vectors for directly gene transfer and to aid in the characterization of the cells, track the cells and genetically modify the cells to express beneficial proteins to enhance their transplantation efficiency. Such as Decorin, mini-dystrophin, and myostatin propeptide genes etc. were constructed in AAV vectors. As described in following, we produced lots of AAV vector for the aims in this project:

1. AAV2-D(+)-CMV-eGFP
2. AAV6-D(+)-CMV-eGFP
3. AAV8-D(+)-CMV-eGFP
4. AAV9-D(+)-CMV-eGFP
5. AAV8-D(+)-CMV-eGFP
6. AAV8-D(+)-tMCK-eGFP
7. AAV2-D(+)-CB-eGFP
8. AAV2-1060-hrGFP
9. AAV2-1070-hrGFP
10. AAV2-1060-Gluciferase
11. AAV2-1070-GLuciferase
12. AAV2-CMV-sflt1
13. AAV2-D(+)-BMP2-spA
14. AAV2-D(+)-BMP2-SV40
15. AAV2-D(+)-CMV-TGFβ1
16. AAV2-CMV-minidystrophin (3858)
17. AAV2-dMCK-minidystrophin (3858)
18. AAV6-D(+)-CMV-BMP4
19. AAV2-D(+)-CMV-VEGF
20. AAV1-CMV-Decorin

Development and production of lenti-viral vectors

In the meanwhile, we also updated the BSL-2 to BSL-2+ for development of lenti-viral vectors using in the aims of this project, including green (GFP) and red (RFPN) reporter genes and silencing lenti-viral vectors targeting to human and mouse VEGF genes.

1. lenti-CMV-GFP
2. lenti-CMV-RFPN
3. lenti-CMV-Luciferase
4. lenti-minidytrophin (3849)-GFP
5. lenti-shRNA-mouse VEGF-GFP
6. lenti-shRNA-human VEGF-GFP

Efficiency of viral vectors

To test the efficiencies of AAV and lenti-viral vectors, we made different viral vectors and the sequential dosages of reporter viral vectors to test which viral vectors could achieve the higher transduction efficiency and lower dose vector loading, such as green fluorescent protein (GFP) and red fluorescent protein in nucleolus (RFP-N), as shown in **Figure 3**. Also, we used same strategy to optimize the serotype of AAV vector that appears a variety of AAV transduction in the different cells and tissues. The advantage of the GFP or RFP reporter is that it can be directly visualized on the whole limb as well as on the cryo-sections. Its fluorescent intensity could also be quantitated by the Nikon microscope imaging software we have been using in the lab. Usually, at 3 weeks following implantation, at which time the GFP gene expression is expected to reach a considerable level for

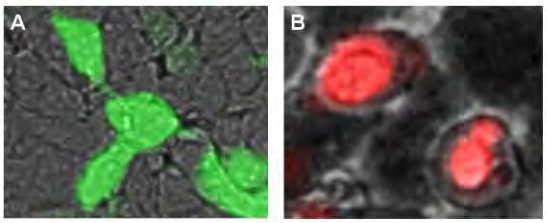


Figure 3. Transduction of AAV- and lenti-viral vectors in 293 cultured cells 36 hours post-infection. (A) shows AAV-GFP, and (B) represents Lenti-RFP-N

detection and quantitation, the animals would be sacrificed and skeletal muscles, hearts and liver will be collected for photographs for GFP or RFP expression and for microscopic photographs and quantitations from the intensity of the fluorescence after cryo-thin-section.

To achieve *ex vivo* gene transfer into *mdx* skeletal muscle using lenti-minidystrophin gene, we constructed a fusion protein containing minidystrophin and GFP reporter gene. GFP reporter can monitor the proliferation and differentiation of muscle derived stem cells *in vivo*, and it can also represent the expression of mini-dystrophin gene directly under microscopy, without toxicity and immune response. In preprimary study, we created this fusion protein construct in a pEGFP-N1 plasmid, and then cloned it into lenti-viral vector under the control of the CMV promoter. As shown in **Figure 4**, the fusion protein was expressed in both C2C12 and 293 cells after transfection.

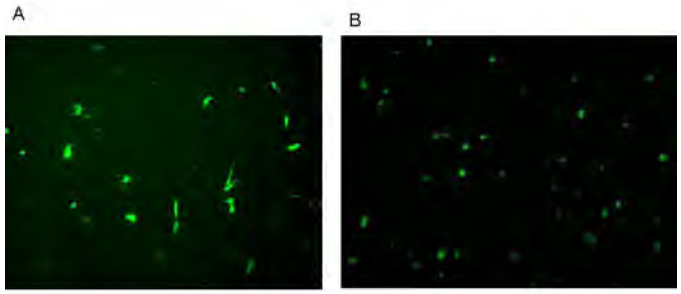


Figure 4. The expression of mini-dystrophin-GFP fusion protein after transfection. (A) 24 hours post-transfection of Mini-dystrophin-GFP fusion in C2C12 by Biotex. (B) 4 days post-transfection of Lenti-Mini-dystrophin-GFP fusion in 293 cells,

Achievements

Invited review book ~~–~~Regenerative therapy for the musculoskeletal system using recombinant adeno-associated viral vectors"

1. **Bing Wang** and Johnny Huard, 2008, the Chapter ~~–~~Gene therapy for the treatment of muscle disorders"
2. **Bing Wang** and Freddie H. Fu, 2008, the Chapter ~~–~~rAAV vectors for soft tissue reconstruction"

Publication and abstracts in 2008 and 2009

1. Chunping Qiao et al. *Human Gen Therapy, 2008, 19 (3), 241-253.*
2. Bing Wang et al. *Gene Therapy, 2008, 15 (15), 1099-1106.*
3. Bing Wang et al. *Gene Therapy, 2008, 15 (22), 1489-1499, correspondence author.*
4. Juan Li etal. *Hum Gene Ther. 2008, 19:958-964.*
5. Bing Wang et al. *Journal of Orthopaedic Research, 2009,27:421-426, correspondence author.*
6. Bin Li et al. *Journal of Biomechanics, 2008, 41:3349-3353.*
7. Michael Y. Mi et al. *Molecular Therapy, 2008, vol.16, suppl 1, S103, correspondence author.*
8. Peiqi Hu et al. *Molecular Therapy, 2008, vol.16, suppl 1, S201*
9. Bhanu M et al. *Molecular Therapy, 2008, vol.16, suppl 1, S293*
10. Joseph Kornegay et al. *Molecular Therapy, 2008, vol.16, suppl 1, S374*
11. Ying Tang; Bin Li, James H-C Wang, Johnny Huard, Melessa Salay, Bing Wang. *In vitro* AAV-mediated gene transfer in human fibroblasts. *54th Annual Meeting of Orthopaedic Research Society, March 1st – March 6th, 2008*
12. Bin Li, Micheal Lin, Ying Tang, Bing Wang, James H-C Wang. Micropatterned C2C12 cells exhibit enhanced differentiation into myotubes – a novel study using cell traction force microscopy. *54th Annual Meeting of Orthopaedic Research Society, March 1st – March 6th, 2008*
13. Guangheng Li, Bo Zheng, Andres J. Quintero, Arvydas Usas, Bing Wang, Laura B. Meszaros, Karin A. Corsi, Johnny Huard. Therapeutic Utility of Heterotopic Ossification Induced by AAV-BMP4 in Skeletal Muscle. *54th Annual Meeting of Orthopaedic Research Society, March 1st – March 6th, 2008*
14. Mi, Y.M; Tang, Y; Li, G-H; Salay, M.N; Niyibizi, C; Huard, J; Wang, B. AAV Based *Ex Vivo* Gene Therapy in Rabbit Adipose Stem/Progenitor Cells for Osteogenesis. *55th Annual Meeting of Orthopaedic Research Society, February 22 – 25, 2009, Las Vegas, Nevada.*

15. Tang, Y; Reay, D.P; Salay, M.N; Mi, Y.M; Clemens, P.R; Guttridge, D.C; Robbins, P.D.; Huard, J; Wang, B. Delivery of Dominant Negative IKKs by Recombinant Adeno-associated Virus: Efficient Improvement in Muscle Regeneration in *mdx* Mice. *55th Annual Meeting of Orthopaedic Research Society, February 22 – 25, 2009, Las Vegas, Nevada.*

16. Michael Y. Mi, Ying Tang, Melisa N. Salay, Guangheng Li, Johnny Huard, Freddie H. Fu, Christopher Niyibizi and Bing Wang. *AAV ex vivo Gene Therapy for Osteogenesis and Chondrogenesis. Submitted to 12th Annual meeting of ASGT (May 27-30, 2009, San Diego, California).*

17. Daibang Nie, Dong Wei, Michael Y. Mi, Ying Tang, Allan Z. Zhao, Yifan Dai, Johnny Huard, and Bing Wang. Muscle Specific Gene Transfer of the PEPCK-C Improves Physical Activity in Mice. *Submitted to 12th Annual meeting of ASGT (May 27-30, 2009, San Diego, California).*

18. Ying Tang, Daniel P. Reay, Melessa N. Salay, Michael Y. Mi, Paula R. Clemens, Denis C. Guttridge, Paul D. Robbins, Johnny Huard, Bing Wang. AAV Based Blocking of NF-kappa B Pathway Improves Muscle Regeneration in *mdx* Mice. *Submitted to 12th Annual meeting of ASGT (May 27-30, 2009, San Diego, California).*

Awards

1. Ying Tang et al. *In vitro* AAV-mediated gene transfer in human fibroblasts. Oral presentation given at the 54th Annual Meeting of Orthopaedic Research Society, March 1st – March 6th, 2008.

2. Michael Y. Mi et al. AAV Based *Ex Vivo* Gene Therapy in Rabbit Adipose Stem/Progenitor Cells for Osteogenesis" was accepted as a podium presentation at the 55th Annual Meeting of Orthopaedic Research Society, February 22 – 25, 2009, Las Vegas, Nevada.

Conclusion

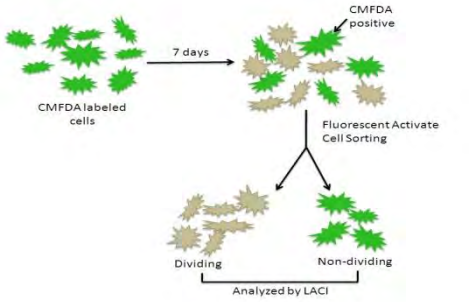
The Vector Core for this project provided a series of viral vectors containing reporter and therapeutic genes proposed in aims. Also, it will develop the Tet-On retro-viral vector system to express a VEGF-LacZ fusion protein.

Bioreactor Core Final Report
(Johnny Huard and Bridget Deasy)

Progress made to date (3-1-06 to 12-31-11):

In 2010, the Bioreactor Core continues to offer unique technology to members of the stem cell research community at the University of Pittsburgh. Here we include another example of how this technology is being used to understand stem cells is below. The project is title, ―Methods to identify and Isolate Potent Human Muscle Derived Stem Cells", TA Bissell, SM Chirieleison and BM Deasy. Following this example, we include a list of publications and abstracts that have used this technology. We also list awards associated with the use of this technology.

The objective of the project ―Methods to identify and Isolate Potent Human Muscle Derived Stem Cells" is to investigate the presence of quiescent cells in muscle derived stem cells (MDSCs) by using live cell imaging. Using FACs and LACI we 1.) directly identified quiescent cells and 2.) isolated the quiescent subpopulation and 3.) verified that the quiescent subpopulation re-activates/ re-enters the cells cycle to give rise to new progeny. Stem cell biology requires an understanding of stem cell population heterogeneity so that the stem cells can be used effectively for regenerative medicine. Stem cell populations contain dividing and non-dividing subpopulations. Among the non-dividing fraction are quiescent cells, for which no definitive markers exists, are cells that are not currently dividing, but have the potential to actively start proliferating. Because of their ability to actively proliferate and give rise to multipotent progeny, the quiescent cells may be the most effect subpopulation for regenerative medicine. Retrospective analysis of previous studies has shown that muscle stem cell populations that contained a larger percentage of quiescent cells contributed to more muscle regeneration (1-3). Further, during stem cells transplantation studies, it has been found that only a small fraction of cells that survive the transplantation process and contribute to regeneration. Live automated cell imaging (LACI) allows human muscle derived stem cells to be observed in a controlled environment for extended periods of time. LACI is a useful tool to classify the subpopulations because it allows the cells to be tracked to determine divisional status and hierarchical lineage. Through the construction of cell lineages and parameters using LACI, the quiescent subpopulation can be identified and studied for its role in cell therapeutics.



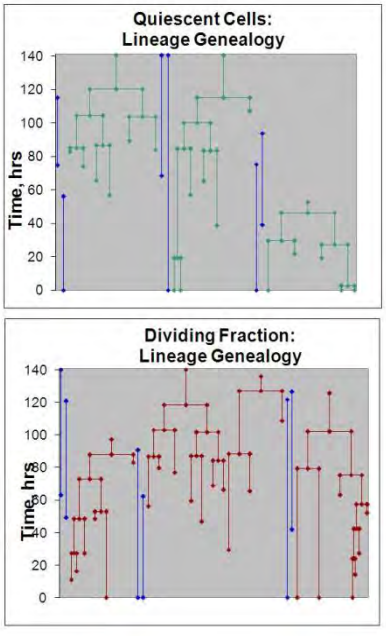


Figure 2. Representative lineage trees from the non-dividing, CMFDA positive subpopulation **(A)** and trees from the dividing, CMFDA negative subpopulation **(B)**. Blue lines represent non-dividing cells, which were on screen for over 40 hours (twice the average DT). The green lines represent quiescent cells that have become activated and begun to divide, and red lineage trees show division for up to five generations

The cells were labeled with a fluorescent cytoplasmic dye, 5-chloromethylfluorescein diacetate (CMFDA). CMFDA decreases in concentration and intensity after a cell divides because the signal is split between the daughter cells. After the cells were in culture for five days, flow cytometry was used to separate the cells into CMFDA positive (non-dividing) and CMFDA negative (dividing) subpopulations. PI cell cycle analysis was performed on the parent population (Figure 1).

The images were analyzed for behavioral parameters and cell lineages.. Cell were tracked and divisions were recorded in order to determine cell histories and to

construct lineage trees. Cell division time, population doubling time and mitotic fraction was determined using the images. (Figure 2-4).

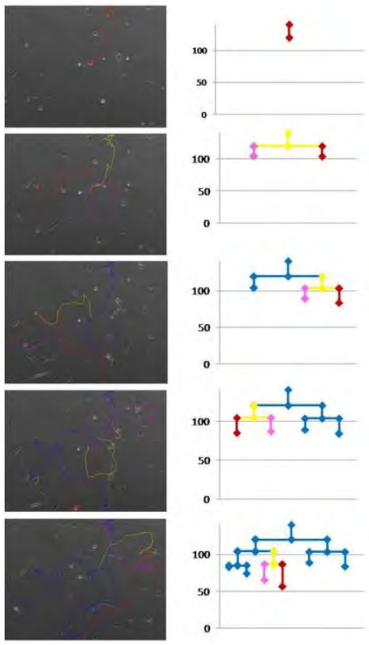


Figure 3. The images represent the tracks and lineage from a re-activated quiescent cell with 4 generations. The images follow the tracks of the parent cell and the daughter cells from the beginning of the video until the cells were lost to follow up. The color of the lineage trees corresponds to the color of the cell tracks. The vertical length of the lines represent the length of time the cell was on screen and the cell division time.

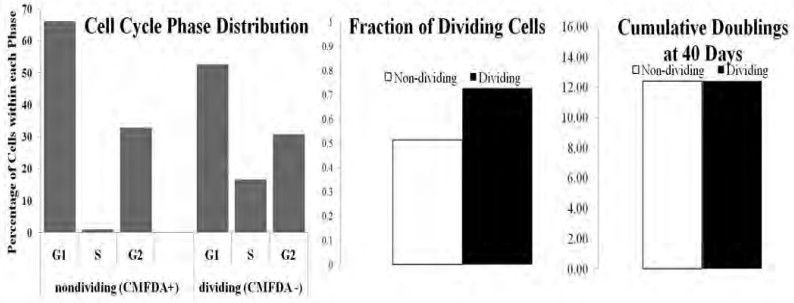


Figure 4. PI cell cycle analysis showed more non-dividing cells in G1 phase. The dividing cell population had a higher percent of dividing cells. In the non-dividing population, 61% of the cells did not divide until after 4 days. 50% of the dividing cells began dividing within the first 6 hrs. In a in-vitro expansion, both populations had 12.4 doublings after 40 days.

In sum, this study showed (1) Labeling the cells with CMFDA and sorting the cells with flow cytometry allows for the quiescent subpopulation to be isolated and identified. (2) Live cell imaging allowed the isolation of the quiescent subpopulation to be validated. The quiescent subpopulation took longer time then the dividing population to begin dividing. (3) The quiescent cells did re-activate and enter the cell cycle. Cell lineages with four generations were observed. The in-vitro expansion showed that the subpopulations had the same longevity.

The ability to isolate and identify the subpopulations allows for a more in depth understanding of stem cell heterogeneity. Regenerative medicine techniques require using cells that can survive the transplantation process and contribute to regeneration. These methods allow the younger quiescent subpopulation to be studied, and provides a method of examining the relationship between cell therapies and tissue repair.

Key Core Users and Collaborative Projects (a sample of several core collaborations)

1. Dr. Bridget Deasy, Department of Orthopedic Surgery, University of Pittsburgh Medical Center
2. Dr. Johnny Huard, Department of Orthopedic Surgery, University of Pittsburgh Medical Center
3. Dr. Yong Li, Department of Orthopedic Surgery, University of Pittsburgh Medical Center
4. Dr. Fabrisia Ambrosio Department of Rehabilitation Sciences, University of Pittsburgh Medical Center
5. Dr. Nam Vo. Dept of orthopedic Surgery, University of Pittsburgh

2010-2011 JOURNAL and ABSTRACT PUBLICATIONS which include Bioreactor Core Technology

JOURNAL PUBLICATIONS

A. Chirieleison, SM, Scelfo, CC, Bissell, TA, Anderson, JE, Koebler, D, Yong Li, Deasy, BM. Robotic imaging systems for biological study of dynamic cell behavior., 2010 *ACCEPTED Biotechnology Progress.*

B. **Deasy, BM*, Chirieleison SM, Witt, AM, Peyton, MJ, Bissell, TA.** 2010. Tracking stem cell function with computers via live cell imaging: Identifying donor variability in human stem cells. *Operative Techniques in Orthopedics*. 20 (2):127-135. ***** *corresponding author.*

C. Tea Soon Park, Manuela Gavina, Chien-Wen Chen, Bin Sun, Pang-Ning Teng, Johnny Huard, Bridget M Deasy, Ludovic Zimmerlin, Bruno Peault. Placental Perivascular Cells for Human Muscle Regeneration. *Stem Cells and Development.* doi:10.1089/scd.2010.0354.

D. Leloup L, Shao H, Bae YH, Deasy B, Stolz D, Roy P, Wells A. m-Calpain activation is regulated by its membrane localization and by its binding to phosphatidylinositol 4,5-bisphosphate. *J. Biol. Chem. 2010 285: 33549-33566.*

E. **Chirieleison SM** , Feduska JM, Schugar RC, Sanford SL, Scelfo CC, Askew Y, Deasy BM. 2010. Human Muscle-Derived Cells Isolated by Differential Adhesion Rates Exhibit Differences in Phenotype and Contribution to Skeletal Muscle Regeneration in mdx/SCID model. *Accepted Tissue Engineering.*

F. **Urish, KL, Deasy BM, Huard J.** (2011). Automated Segmentation, Classification, and Visualization of Fluorescent Time-lapsed Microscopy Images. In submission.

ABSTRACT PUBLICATIONS (selected list of > 30)

1. Witt, A M; Nance, J J; James, S D; Usas, A; Deasy, B M. Human Donor Variability in Osteogenesis is a Consideration in the Therapeutic Use of Umbilical Cord. 2010. International Society for Stem Cell Research; San Francisco, CA .
2. Deasy, BM, Bissell, TA, Chirieleison, SM, Piganelli JD. Ex Vivo Expanded Human Muscle Stem Cells show increased levels of reactive oxygen species and increased sensitivity to oxidative stress. The Ottawa Conference on New Directions in Biology and Disease of Skeletal Muscle 2010.
3. Deasy, BM, Feduska JM, Payne TR, Li Y, Ambrosio F, Huard J. Improved muscle repair through transplantation of VEGF-expressing muscle stem cells in mdx skeletal muscle. Ottawa Meeting. The Ottawa Conference on New Directions in Biology and Disease of Skeletal Muscle 2010.
4. Deasy, BM, Chirieleison SM; Witt, AM; Bissell, TA; Peyton, MJ. 2010. Tracking stem cell activity with computers via live cell imaging: Identifying donor variability in human umbilical cord stem cells. *Pittsburgh Orthopedic Journal*
5. Bissell, T A; Chirieleison SM; Witt, M; Kline, D E; Deasy, B M. 2010. Identification of Potent Human Muscle Stem Cells by Live Cell Imaging. *Pittsburgh Orthopedic Journal*
6. Jordan E. Anderson, Steven M. Chirieleison, Christopher C. Scelfo, Taylor A. Bissell, Yong Li , Doug J. Bridget M. Deasy 2010. Robotic imaging systems for biological study of dynamic cell behavior in cell

migration assays. *Pittsburgh Orthopedic Journal*
7. Anderson JE, Yao V, Chu CR, Deasy B. 2010. THE USE OF TIME-LAPSED MICROSCOPY TO STUDY CELL MIGRATION AND CHEMOATTRACTION. *Pittsburgh Orthopedic Journal*
8. Chirieleison S C; Nance, JJ; Schugar, RC; Witt, AM; Deasy, B M. 2010. Sex Differences in In Vivo Skeletal Muscle Regeneration after Transplantation of Human Umbilical Cord Stem Cells to mdx/SCID mice. *Pittsburgh Orthopedic Journal*
9. Nance, JJ; Wescoe, KE; Witt, AM; Deasy, B M. 2010. Induction of Chondrogenesis in Human Umbilical Cord Derived Stem Cells in a Hypoxic Environment. *Pittsburgh Orthopedic Journal*
10. Witt, AM Nance, JJ; James, SD; Wescoe, KE; Usas, A; Deasy, B M. 2010. Human Donor Variability in Osteogenesis of Human Umbilical Cord Stem Cells. *Pittsburgh Orthopedic Journal*
11. Friesmuth, KR Payne, KA; Schugar, RC, Chu, CR; Deasy, B M Comparison of Expandability of Mesenchymal Cells from Newborn Umbilical Cord and Adult Bone Marrow. *Pittsburgh Orthopedic Journal*
12. Nance, J J; Wescoe, K E; Witt, A M; Deasy, B M Induction of Chondrogenesis in Umbilical Cord Derived Stem Cells in Hypoxic Environment. 2010 Orthopaedic Research Society; New Orleans, LA
13. Bissell, T A; Chirielsion S C; Witt, M; Kilne, D; Deasy, B M. Potent Human Muscle Stem Cells are Identified by Live Cell Imaging. 2010 Orthopaedic Research Society; New Orleans, LA
14. Chirieleison, S M; Nance, J J; Schugar, R.C. Witt, A.M. Deasy, B M Sex Differences in In Vivo Skeletal Muscle Regeneration after Transplantation of Human Umbilical Cord Stem Cells to mdx/SCID mice. 2010 Orthopaedic Research Society; New Orleans, LA

AWARDS and OTHER ACCOMPLISHMENTS

1. **A new NIH award** STTR 1R41AR057629-01 –Processing and Preservation of Human Umbilical Cord Stem Cells for Cell Therapeutics" to BM Deasy was received and is based in part on the live cell imaging technology to facilitate the identification of stem cells processing standards.

2. Two University of Pittsburgh Bioengineering undergraduate student were awarded fellowships related to their projects which use live cell imaging:
   1. **Jordan E. Anderson**- Honors Research Assistantship, University of Pittsburgh. Mentor: Dr. BM Deasy
   2. **Taylor A. Bissell** - Chancellor's Undergraduate Research Fellowship Univ of Pittsburgh. Mentor: Dr. BM Deasy

3. The LiveCell Imaging Lab technology was featured at the annual Orthopedic Research Society Meeting in 2011 at a special ***Spotlight on Research*** workshop.

Micro-CT Core Final Report
(Johnny Huard and Arvydas Usas)

Progress made to date (3-1-06 to 12-31-11):

We continued to use the vivaCT 40 (Scanco Medical) imaging system for nondestructive 2-D and 3-D visualization and quantitative analysis of mineralized matrix volume, density and other structural parameters of bone tissue. VivaCT 40 system enables us to perform live animal imaging on the same animal at different time points; therefore we are able to reduce the number of experimental animals and cut the cost for animal housing and care. We also performed CT imaging of various animal body parts and tissues (calvaria, spine, extremities, muscles, etc.) harvested after animal euthanasia and stored in fixative solution for extended period of time.
We used continuous cell pellet culture imaging to detect matrix mineralization after osteogenic stimulation in vitro. This system we believe can be very useful for the screening of cellular candidates for bone tissue engineering.
We began new studies where we use microCT for evaluation of bone in various animal models of accelerated aging (dkO mice, *Ercc1*-deficient mice, and Zampste24 metalloproteinase-deficient mice). We will evaluate various parameters of bone structure in these animals. We will also create calvarial and long bone defect in these mice and evaluate bone healing. After this we will inject muscle-derived stem cells in aged animals in order to improve bone formation and enhance bone healing. We are planning to initiate microCT for cartilage imaging using various contrast agents.

During the report period the use of micro-CT contributed to the following research accomplishments by the members of our laboratory:

1. MDSCs promote bone healing by contributing directly to bone regeneration and by inducing early and enhanced inflammation and angiogenesis. Gao X, et al. NIRA poster presentation at the ORS meeting 2011, Long Beach, CA.
2. Repair of Non-Healing Bone Defect by Muscle-Derived Stem Cells. Podium presentation at the ORS meeting 2011, Long Beach, CA.
3. Effect of Sex Hormones on Bone Defect Repair by Muscle-Derived Stem Cells. Poster presentation at the ORS meeting 2011, Long Beach, CA.
4. Dynamic observation revealed muscle derived stem cells transplanted in the cranial skull defect induce early inflammatory response and angiogenesis to promote bone healing. Gao X, et al. Poster presentation at the ORS meeting 2011, Long Beach, CA.
5. The Influence of Platelet-Rich Plasma on In-vitro Proliferation, Osteogenic, Chondrogenic, and Myogenic Differentiation of Human Muscle Derived Progenitor Cells. Li H, et al. Poster presentation at the ORS meeting 2011, Long Beach, CA.
6. Alkaline Phosphatase Expressing Human Skeletal Muscle-Derived Cells Exhibit Distinct Osteogenic Differentiation Potential In Vitro and In Vivo. Usas A, et al. Poster presentation at the ORS meeting 2011, Long Beach, CA.
7. Pericyte-based human tissue engineered vascular grafts. He W, et al. Biomaterials. 2010 Nov;31(32):8235-44.
8. Accelerated aging of intervertebral discs in a mouse model of progeria. Vo N, et al. J Orthop Res. 2010 Dec;28(12):1600-7.
9. Role of PI3K, ERK1/2, and p38 MAPK pathways in BMP4-induced matrix mineralization by MDSCs. Payne-Corsi K, et al. Manuscript accepted for publication in Tissue Eng.
10. Inhibition of NF-kB delays the onset of age-related degenerative diseases. Tilstra JS, et al. Manuscript in preparation for submission.

11. Isolation and characterization of human ACL-derived vascular stem cells. Matsumoto T, et al. Manuscript submitted to Cell Transplantation.
12. Myoendothelial cells: a novel stem cell population from adult human skeletal muscle. Zheng B, et al. Manuscript submitted to Journal of Molecular Cell Biology.
13. Identification and characterization of chondrogenic progenitor cells in adult skeletal muscle. Li GH, et al. Manuscript submitted to Journal of Molecular Cell Biology.
14. Osteogenic potential of male and female human skeletal muscle derived cells. Corsi-Payne K, et al. Manuscript in preparation.
15. Human skeletal muscle cells can undergo matrix mineralization in vitro and facilitate bone repair after delivery on SIS scaffold. Usas A, et al. Manuscript in preparation.
16. The donor-host cell interaction in muscle stem cell based bone regeneration. Gao X, et al. Manuscript in preparation.

Following is the list of current research projects that involve the use of micro-CT core:

1. Osteogenic potential of different populations of human muscle-derived cells, sorted by FACS for high and low ALP, ALDH expression, before and after transduction with BMP4 (NIH funded project).
2. Interaction between donor and host cells in muscle-derived stem cell mediated bone formation (NIH grant proposal).
3. Evaluation of bone structure and bone defect healing in different animal models of accelerated aging (In collaboration with Drs. B. Wang, M. Lavasani, L. Niedernhofer).
4. The role of inflammation and angiogenesis in bone formation in different animal models of accelerated aging (NIH grant proposal).

Administrative Core Progress Report
(Johnny Huard, James Cummins and Matthew Bosco)

INTRODUCTION:

The Administrative Core of the Stem Cell Research Center (SCRC) was directly responsible for ensuring the proper function and integration of the Research Laboratories (comprising the Core Research Laboratories, Affiliated Laboratories, and Research Core Facilities), the Clinical Trials Unit, and the Educational Programs that constitute the SCRC. The Administrative Core provided administrative services to all SCRC personnel, supports the ongoing activities of the SCRC, and provides a mechanism for regular evaluation of the SCRC. The Administrative Core was also responsible for fulfilling the secretarial, budgetary, and grant application and manuscript preparation needs of SCRC personnel. In addition, this Core facilitated collaboration between SCRC researchers and scientists working in designated Collaborative Institutes or other, non-affiliated laboratories.

BODY:

The Administrative Core provided managerial and financial oversight for the entire Department of Defense Program. The center administrator, Matthew Bosco, and Senior Scientist, James Cummins played key roles in facilitating and administering all of the various projects within the Program. Mr. Bosco and Mr. Cummins helped ensure that each project functioned smoothly and efficiently while maintaining continuous communication between the various investigators and institutions.

The Administrative Core held weekly/biweekly seminar series for SCRC researchers, affiliates, collaborators, and other interested scientists. The goal of each of these events was to bring in highly regarded scientists who performed cutting edge research in cellular therapeutics. In addition to serving as a forum in which SCRC scientists can interact with other researchers who share similar interests, this series helped to introduce invited speakers to the SCRC and helped to promote the development of ongoing collaborations with these well-regarded research scientists. The goal of these meetings was to help SCRC researchers hone their scientific critical analysis skills and, by so doing, improve their own research and writing abilities.

The use of suramin to improve skeletal muscle healing after contusion injury

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*Stem Cell Research Center, Children's Hospital of Pittsburgh and Department of Orthopaedic Surgery, University of Pittsburgh, Pittsburgh, PA
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INTRODUCTION:

Muscle injuries are very common musculoskeletal problems encountered in sports medicine. Although this type of injury is capable of healing, an incomplete functional recovery often occurs, depending on the severity of the blunt trauma. Complete functional recovery is hindered by the development of scar tissue formation, which typically appears during the second week following muscle injury. We have reported that TGF-β1 is a major factor in triggering the fibrotic cascade within injured skeletal muscle [1]. The use of antifibrosis agents, such as suramin, that inactivates TGF-β1 can reduce muscle fibrosis and consequently improve muscle healing after injury. The ability of suramin to block the fibrotic effect of TGF-β1 and reduce the fibrosis makes this molecule well suited for use in applications to improve muscle healing after injury. We already reported that suramin can effectively prevent muscle fibrosis and enhance muscle regeneration in the lacerated and strain-injured muscle [2,3]; however, it is still not known if suramin can improve muscle healing after contusion injury, which is the most commonly encountered muscle injury. Furthermore it has remained unclear whether this enhanced muscle regeneration is a direct effect of suramin. We performed this study to examine whether suramin would promote differentiation of myogenic cells *in vitro* and improve injured muscle healing by enhancing regeneration and reducing fibrosis *in vivo*, using an animal model of muscle contusion.

MATERIALS AND METHODS:

Muscle-derived stem cell differentiation assay: Muscle-derived stem cells (MDSCs) were isolated from wild type mice(C57BL/6J) via the modified preplate technique [4]. MDSCs (10^4 cells/well) were seeded into 12 well plates and were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum, 10% horse serum, 0.5% chicken embryo extract, and 1% penicillin/streptomycin. After 24 hours, the medium was replaced with differentiation medium (DMEM containing 2% horse serum and 1% penicillin/streptomycin) containing different concentration of suramin (0, 1, 10, 100μl/ml). After another 24 hours, the medium was replaced with only differentiation medium. All cells were grown at 37°C in 5% CO_2. Three days after incubation the fusion index was assessed by counting the number of nuclei in differentiated myotubes as a percentage of the total number of nuclei.

Immunocytochemistry in vitro: Immunocytochemistry was performed on the cells *in vitro* to examine their expression of fast myosin heavy chain (MyHC). Mouse anti-MyHC (1:250; Sigma) was used as the primary antibody, and biotinylated anti-mouse IgG (1:200; Vector) was used as the secondary antibody. Streptavidin 555 conjugate (1:500; Molecular Probes) was applied to detect the secondary antibody.

Animal model: The muscle contusion model was developed in normal wild-type mice (7-10 weeks, average weight 24.0g). A 17g stainless steel ball was dropped through an impactor from a height of 100cm onto the animal's tibialis anterior (TA) muscle. Mice were divided into 4 groups (5mice/group). Different concentrations of suramin (0, 2.5, 5, 10mg in 20μl of Phosphate-buffered solution [PBS]) were injected intramuscularly two weeks after injury. Cryostat sections of muscles (10μm in thickness) were obtained and histologically stained (hematoxlin and eosin stain (H&E) and Masson's Trichrome stain) four weeks after injury. The numbers of the centronucleated regenerating myofibers from each group was determined to evaluate the regeneration. Northern Eclipse software (Empix Image, Inc.) was used to quantify the total fibrotic area. Statistical analysis was performed with ANOVA.

RESULTS:

Suramin stimulates MDSCs's differentiation: Suramin treatment promoted the differentiation of MDSCs *in vitro* in a dose dependent manner. We observed a significantly higher fusion index in each of the two suramin treatment groups (10 and 100μg/ml) than in the control group (0μg/ml). Furthermore, 100μg/ml of suramin treatment enhanced the differentiation significantly more than the other suramin treatments (1 and 10μg/ml) (Fig.1,2).

Suramin enhances muscle regeneration and decreases fibrosis after contusion injury: We observed a significant increase in the number of regenerating myofibers in all of suramin treated groups (2.5, 5, 10mg/20μl PBS) when compared with the control group (0mg/20μl of PBS) (Fig. 3,4). Moreover Masson's Trichrome staining showed significantly less fibrotic area in all of suramin treated groups than in the control group (Fig. 5, 6). Although all three suramin treated groups showed significant improvement in healing by way of muscle regeneration and fibrosis inhibition, there was no significant difference between the three suramin treatment groups (data not shown).

Figure 1. Immunocytochemistry of MyHC

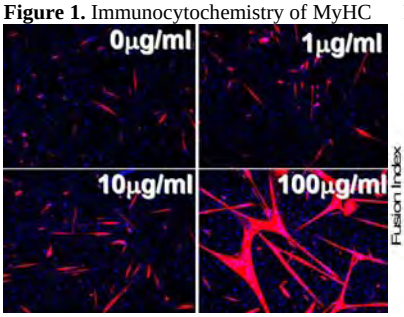


Figure 2. Fusion Index

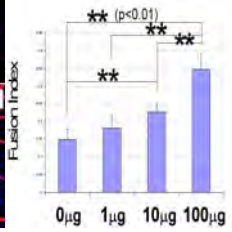


Figure 3. H-E staining 4 wks after contusion

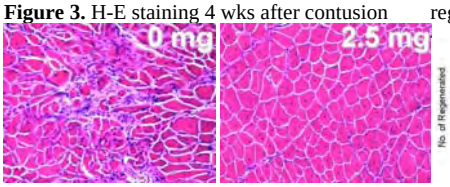


Figure 4. Number of regenerated myofibers

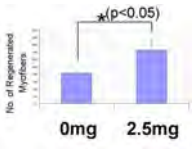


Figure 5. Trichrome staining after contusion

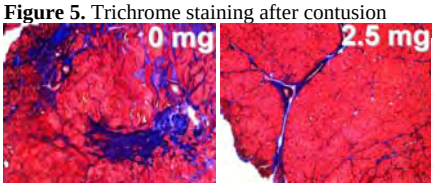
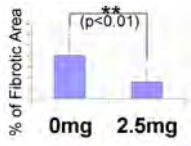


Figure 6. Percentage of Fibrotic area



DISCUSSION:

We have reported that suramin can effectively prevent muscle fibrosis and enhance muscle regeneration after laceration and strain injury. Furthermore, our preliminary data has indicated that suramin blocks the proliferative effect of TGF-β1 on fibroblasts *in vitro* [2,3]. We examined the effect of suramin on the differentiation of MDSCs after we observed that suramin enhanced the differentiation of C2C12 as a preliminary study. Our results from this experiment showed that the suramin treated MDSC groups have higher fusion indices than the control group in vitro. This indicates that suramin can enhance the differentiation of MDSCs, revealing part of the mechanism by which suramin enhances the muscle regeneration after injury. This is the first study to show that suramin is affecting the differentiation of MDSCs directly in addition to it's antiproliferative effect on fibroblasts. Furthermore, our result shows suramin can enhance muscle regeneration and prevent fibrosis after a contusion injury, the most common muscle injury. Our future study will investigate the mechanism by which suramin enhances the differentiation of myogenic cells.

Acknowledgements:

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Appendix 2

Decorin Gene Transfer Promotes Muscle Cell Differentiation and Muscle Regeneration

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We have shown that decorin, a small leucine-rich proteoglycan, can inhibit transforming growth factor (TGF)-β1 to prevent fibrous scar formation and improve muscle healing after injury. In the decorin-treated muscle, an enhancement of muscle regeneration is observed through histological examination. In this article, we report our determination of whether decorin has a direct effect on myogenic cells' differentiation. Our results indicate that myoblasts genetically engineered to express decorin (CD cells) differentiated into myotubes at a significantly higher rate than did control myoblasts (C2C12). This enhanced differentiation led to the up-regulation of myogenic genes (*Myf5*, *Myf6*, *MyoD*, and myogenin) in CD cells in vitro. We speculate that the higher rate of differentiation exhibited by the CD cells is due to the up-regulation of follistatin, peroxisome-proliferator-activated receptor-gamma co-activator-1α (PGC-1α), p21, and the myogenic genes, and the down-regulation of TGF-β1 and myostatin. Decorin gene transfer *in vivo* promoted skeletal muscle regeneration and accelerated muscle healing after injury. These results suggest that decorin not only prevents fibrosis but also improves muscle regeneration and repair.

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INTRODUCTION

Decorin, a small leucine-rich proteoglycan, is a component of the extracellular matrix of all collagen-containing tissues.[1] Decorin is pivotal in regulating the proper assembly of collagenous matrices and in controlling cell proliferation under various conditions.[2] On the basis of its ability to bind fibrillar collagen and delay *in vitro* fibrillogenesis, decorin is regarded as a key modulator of matrix assembly.[3,4] This proteoglycan can modulate the bioactivity of growth factors and act as a direct signaling molecule to different cells.[5] Decorin, which is expressed at high levels in skeletal muscle during early development,[6] also interferes with muscle cell differentiation and migration and regulates connective tissue formation in skeletal muscle.[7–9]

Because terminal differentiation is critical for initial skeletal muscle development and regeneration after injury and disease,[10] we examined decorin's role in remodeling healing skeletal muscle. We have shown that the direct injection of bovine decorin decreased muscle fibrosis and provided nearly complete functional recovery.[11] Decorin blocks fibrosis (mostly by inhibiting transforming growth factor (TGF)-β activity), which improves muscle healing. However, the role of decorin in muscle cell differentiation and regeneration is still unknown. Although we hypothesize that decorin's effect on muscle fibrosis may indirectly impact regeneration, we were unable to exclude the possibility that decorin promotes regeneration independent of its effects on fibrosis formation.

Many studies have investigated the mechanism behind the antifibrotic effect of decorin.[3,12–14] Others have shown that hepatocyte growth factor increases decorin production by fibroblasts through the extracellular signal–regulated kinase 1/2, and p38 mitogen-activated protein kinase–mediated pathways.[14] Decorin stimulates the growth of smooth muscle cells under specific conditions and influences the growth of epidermal cells by interacting with epidermal growth factor and its receptors.[15,16] Recent research has shown that decorin can bind both insulin-like growth factor-I and its receptor; this interaction leads to the phosphorylation of protein kinase B (Akt) and p21 expression in endothelial cells.[17]

Decorin also influences muscle cell behavior by interacting with p21, an important cyclin-dependent kinase inhibitor.[18,19] Follistatin and myostatin are involved in the control of muscle mass during development. These two proteins have opposite effects on muscle growth, as documented by genetic models.[21,22] Recent studies have shown that myostatin action is inhibited by decorin,[23] resulting in enhanced healing and reduced fibrosis within myostatin-null mice compared with wild-type mice.[24] A recent study indicates that peroxisome-proliferator-activated receptor-gamma co-activator-1α (PGC-1α), is also involved in the muscle healing process and influences muscle fiber–type determination.[25,26] Decorin may also interact with PGC-1α expression in skeletal muscle after injury.

In this study, we investigated the *in vitro* effect of decorin on the differentiation of myoblasts (C2C12) and characterized the *in vitro* and *in vivo* behavior of myoblasts transfected with the

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decorin gene (CD cells). We also studied the influence that decorin over-expression had on myostatin, follistatin, PGC-1α, and p21 expression. Using an adeno-associated virus (AAV) vector, we transduced the decorin gene into injured skeletal muscle to further investigate its function on muscle healing. Our overall goal in this study was to determine whether decorin could improve skeletal muscle healing by enhancing muscle regeneration independently of its antifibrotic action.

RESULTS

Genetic engineering of myogenic cells to over-express decorin

We used lipofectin to transfect a pAAV-CMV-decorin plasmid (**Figure 1a**) into both 293 cells (packaging cell line) and C2C12 cells (myoblast cell line). The results of western blot analysis (**Figure 1b**) showed decorin in both the supernatant (culture media) and the lysate of the 293 cells 48 hours after transfection. The transfected C2C12 cells (CD clone cells) expressed decorin (**Figure 1c:** lane 2, 24 hours; lane 4, 48 hours; lane 5, decorin-positive control), whereas non-transfected C2C12 cells did not (**Figure 1c:** lane 1, 24 hours; lane 3, 48 hours). We also detected decorin in both myoblasts (C2C12) and muscle-derived stem cells after mDecorin-AAV (mDec-AAV) gene transfer *in vitro* (**Figure 1d** and **e**).

Decorin stimulates myoblast differentiation *in vitro*

To investigate myoblast differentiation, we compared decorin-co-cultured C2C12 cells with non-treated C2C12 cells *in vitro*. C2C12 cells cultured with decorin (10 μg/mL) and grown in differentiation/fusion medium exhibited significantly enhanced differentiation and fusion *in vitro*. After 3 and 4 days of stimulating C2C12 cells with decorin, we observed a significant increase in the number of myotubes when compared with un-stimulated C2C12 (control) cells ($P < 0.01$ at 3 days and $P < 0.05$ at 4 days, respectively). However, the numbers of myotubes 5 days after treatment were not significantly different (**Figure 2a**). We then evaluated whether CD cells exhibited a greater propensity to undergo myogenic differentiation than did non-transfected C2C12 cells. As shown in **Figure 2b** (myotubes stained in mouse anti-myosin heavy chain are red) and **Figure 2c**, CD cells generated significantly more myotubes overall and created significantly larger myotubes than did non-transfected C2C12 cells.

Decorin increases myoblast differentiation and induces myogenic gene expression *in vitro*

We investigated whether the CD cells expressed higher levels of myogenic genes than did non-transfected C2C12 (control) cells. Our results, shown in **Figure 3a**, demonstrate that decorin gene transfer led to higher expression of the myogenic genes *Myf5*, *Myf6*, *MyoD*, and *myogenin*. Desmin expression levels in CD and C2C12 cells remained similar.

Decorin up-regulates p21, follistatin, and PGC-1α, but down-regulates TGF-β1 and myostatin in C2C12

We also performed experiments designed to investigate the mechanism by which decorin influences the differentiation of muscle cells. We found that CD cells exhibited increased p21 expression and decreased myostatin expression (**Figure 3b**). The C2C12 cells can be induced to express TGF-β1 in an autocrine manner, as we have previously determined;[27] however, the CD cells do not show any detectable expression of TGF-β1 after

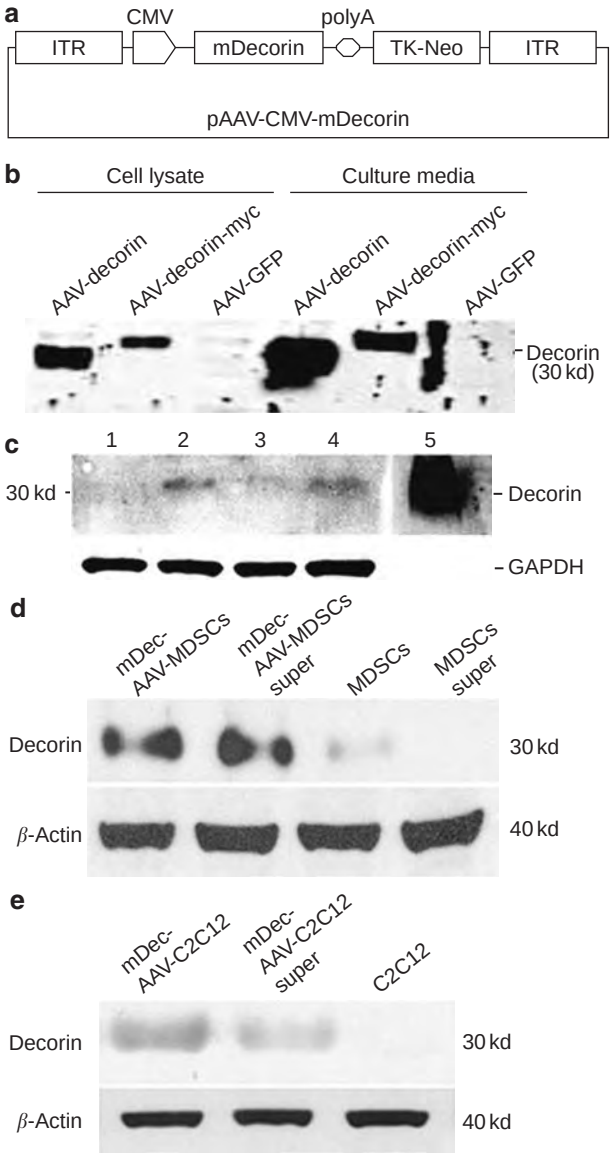

Figure 1 Decorin plasmid construction and initial transfection *in vitro*. (**a**) The decorin plasmid used for the study contained the full sequence of a mouse decorin gene inserted at the *Not*I site, which placed it under the control of a cytomegalovirus (CMV) promoter. (**b**) We transfected the plasmid into 293 cells. We observed decorin expression in both the 293 cells and their supernatant, but not in the control adeno-associated virus (AAV)–transfected (green fluorescent protein, GFP) cells. (**c**) Western blot analysis also revealed decorin expression in CD clone cells within different time period cultures (lane 2, 24 hours; lane 4, 48 hours), but not in C2C12 (lane 1, 24 hours; lane 3, 48 hours). We used 5 μg of decorin as a positive control (lane 5). GAPDH, glyceraldehyde-3-phosphate dehydrogenase is used as a control. (**d**) Decorin expression in muscle-derived stem cells (MDSCs) in pellet form was low, but this was not the case in the supernatant. Both MDSCs and their cultured supernatant strongly expressed decorin after mDec-AAV gene transfer. β-actin is used as a control. (**e**) We did not detect decorin in normal C2C12 cells, but C2C12 cells and their cultured supernatant both expressed decorin after mDec-AAV gene transfer.

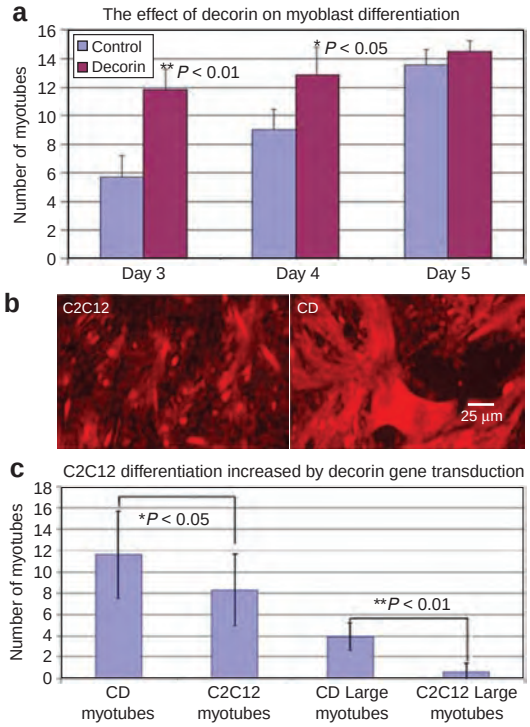

Figure 2 Decorin stimulates C2C12 differentiation *in vitro*. (**a**) Decorin treatment accelerated the differentiation and fusion of myoblasts (C2C12 cells) compared with non-treated myoblasts (C2C12 cells). (**a**) The cultures of decorin-treated C2C12 cells contained more myotubes at the 3- and 4-day time points than control cells. (**b**, **c**) Similarly, decorin-transfected C2C12 clone cells (CD cells) produced more myotubes than did C2C12 cells, including larger myotubes (containing more than three nuclei) *in vitro*. Red staining shows myosin heavy chain fluorescence after immunostaining (**b**).

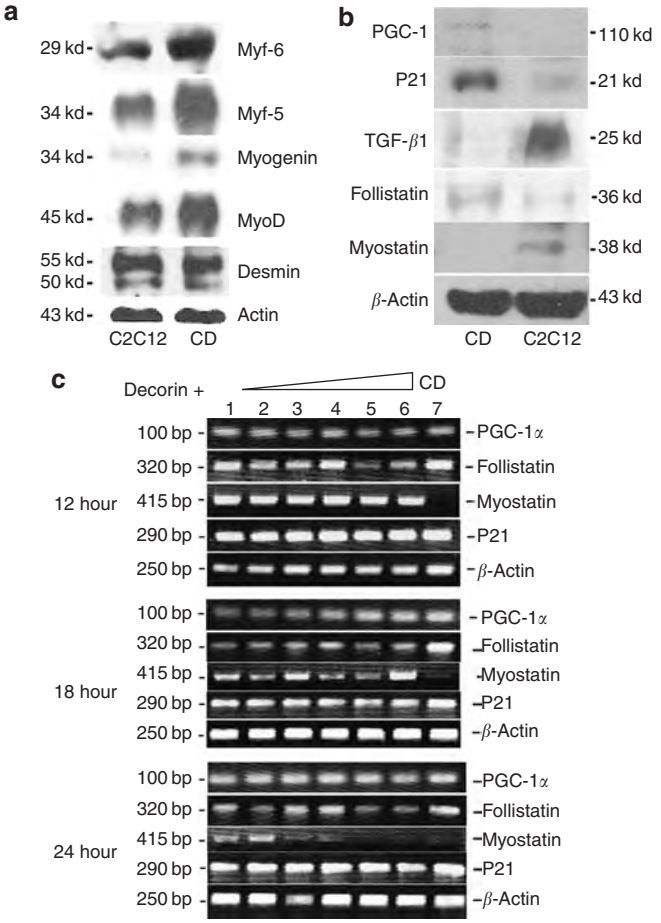

Figure 3 Decorin gene transfer up-regulates myogenic proteins and p21 and down-regulates myostatin during muscle cell differentiation. (**a**) Genetic engineering of myoblasts to express decorin influenced the expression of myogenic proteins (including Myf5/6, Myogenin, and MyoD), as shown by western blot results. However, C2C12 cells and CD clone cells expressed comparable levels of desmin. (**b**) We detected the presence of p21 expressed in CD clone cells. We also detected peroxisome-proliferator-activated receptor-gamma co-activator-1α (PGC-1α) and follistatin expressed in CD, but not C2C12, cells. In addition, CD cells exhibited lower levels of myostatin, a negative regulator of muscle mass. The induction of tumor growth factor (TGF)-β1 auto-expression was also inhibited by decorin over-expression in CD cells. (**c**) Similar results were obtained by real-time polymerase chain reaction. Lanes 1–6 show results for C2C12 cells exposed to different concentrations of decorin (0, 0.001, 0.01, 0.1, 1.0, and 5.0 ng/ml, respectively). Lane 7 displays the test results for CD cells, which served as the positive control. We did not detect a visible change in the expression of PGC-1α, follistatin, myostatin, or p21 after 12 hours of stimulation with different concentrations of decorin. PGC-1α and follistatin were up-regulated in C2C12 cells in a dose-dependent manner after 18 hours of stimulation with decorin. Myostatin was down-regulated in C2C12 cells in a dose-dependent manner after 24 hours of decorin stimulation. The concentration of p21 did not visibly change after cell stimulation with any experimental concentration of decorin over all time points. With decorin gene transfer, we found that CD cells consistently expressed follistatin, p21, and PGC-1α but were negative for myostatin. Note that β-actin was selected as a positive gene control.

TGF-β1 stimulation. More importantly, we detected that both follistatin and PGC-1α (**Figure 3b**) had been up-regulated when compared with C2C12 cells. We also discovered that follistatin and PGC-1α messenger RNA were altered in C2C12 cells after decorin stimulation, as determined by real-time polymerase chain reaction. Specifically, we found that PGC-1α and follistatin increased in a dose-dependent manner after 18 hours of stimulation with decorin, and that myostatin was decreased in a dose-dependent manner after 24 hours of stimulation with decorin (**Figure 3c**). In CD cells, we also observed increased amounts of all three genes (*p21*, *follistatin*, and *PGC-1α*), but a decrease in myostatin was observed (**Figure 3c**).

The up-regulation of *follistatin*, *PGC-1α*, *p21*, and myogenic genes, including *MyoD* (**Figure 3a**), in CD cells could at least partially explain how decorin promotes muscle cell differentiation. Alternatively, the down-regulation of myostatin, a well-known negative regulator of muscle growth during muscle regeneration, could also benefit muscle cell differentiation.

The implantation of CD cells in skeletal muscle results in improved muscle regeneration

The implantation of CD cells within skeletal muscle resulted in significantly better muscle regeneration than that observed for control C2C12 cells, as determined 4 weeks after injection of the cells into *MDX*/SCID mice. Although the number of LacZ-positive muscle fibers (*i.e.*, regenerating muscle fibers) did not differ between the groups (**Figure 4a**, **c**, and **d**), the diameters of the regenerating muscle fibers (*e.g.*, dystrophin-positive myofibers) in the muscles injected with CD cells were significantly larger than those of the regenerating muscle fibers in the control muscles

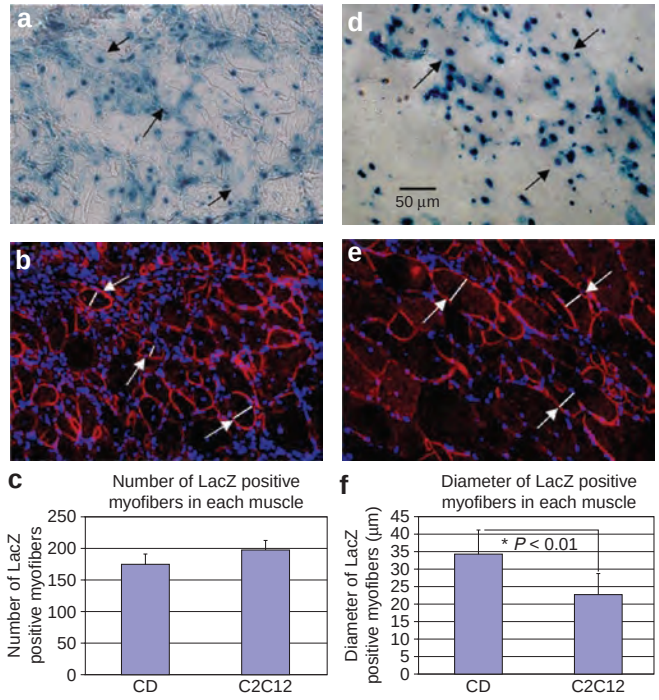

Figure 4 Decorin gene transfer stimulates muscle regeneration *in vivo*. C2C12 cells regenerated muscle fibers after transplantation into skeletal muscle of *MDX*/SCID mice, as shown on both (**a**) LacZ- and (**b**) dystrophin-expressing myofibers. However, transplantation of CD clone cells, rather than C2C12 cells, resulted in larger muscle fibers in *MDX*/SCID mice, as shown in some of the (**d**) LacZ- and (**e**) dystrophin-positive myofibers. (**c**) Although there was no significant difference between the number of LacZ-labeled muscle fibers that formed in muscles transplanted with C2C12 or CD cells, (**f**) the transplantation of CD cells resulted in the regeneration of larger-diameter myofibers ($P < 0.01$).

(**Figure 4b**, **e**, and **f**; dystrophin is red). The larger diameters of the dystrophin-positive muscle fibers generated by CD cells could indicate that implantation of CD cells accelerated muscle regeneration; however, we were unable to exclude the possibility that the CD cells may have a greater propensity to fuse in host myofibers than C2C12 control cells.

mDec-AAV gene transfer promotes muscle regeneration and reduces fibrosis

Better muscle regeneration was observed within mDec-AAV-treated muscle (**Figure 5a** and **b**) than within non-mDec-AAV-treated muscle at 2 weeks after injury (**Figure 5c** and **d**). Histological analysis of total collagen deposition 4 weeks after injury revealed that mDec-AAV-injected muscles contained less fibrous scar tissue in the injured area than did non-treated control muscles (**Figure 5f**, **h**, and **j**; collagen deposition areas are blue). We also observed that decorin stimulated skeletal muscle regeneration 4 weeks after laceration injury. We found that mDec-AAV-injected muscles contained more centronucleated (regenerating) myofibers and less scar tissue 4 weeks after injury than did control muscles (**Figure 5e**, **g**, and **i**).

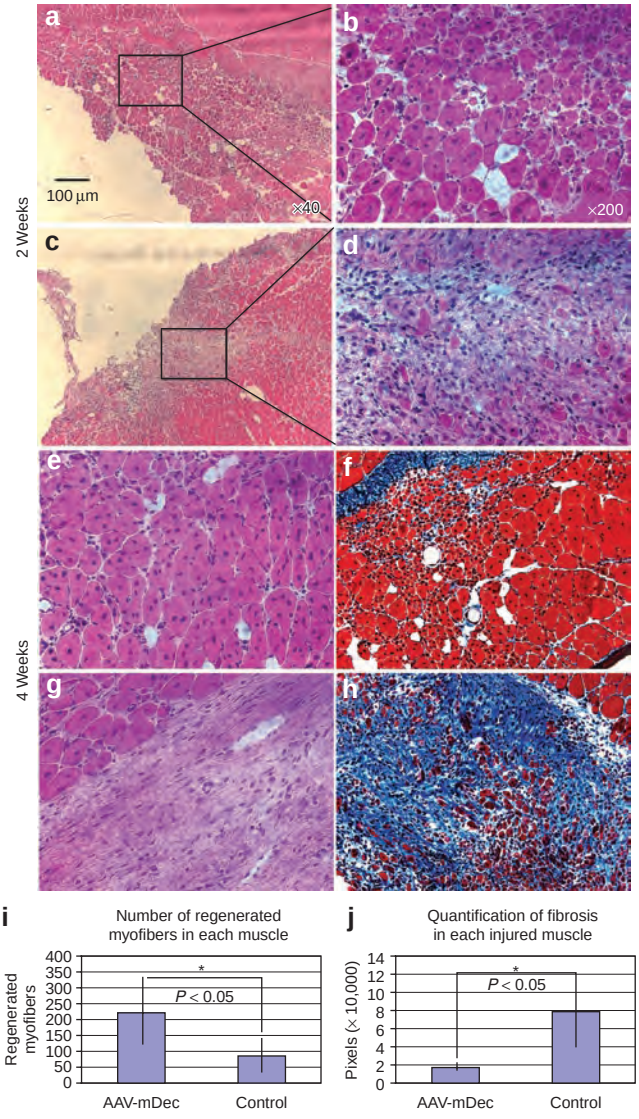

Figure 5 mDec-AAV vector gene therapy in injured muscle prevents fibrosis and promotes muscle regeneration. Decorin-treated muscle exhibits a greater number of regenerating myofibers than the control muscle at all time points (**a–d** 2 weeks; **e–h** 4 weeks). (**i**) The mDec-AAV-injected muscle contained significantly higher numbers of centronucleated (regenerated) myofibers than did control (sham-injected) muscle at 4 weeks after therapy. We also found that decorin gene therapy minimized fibrosis in injured skeletal muscle. We used Masson's trichrome staining to reveal collagen in injured skeletal muscle, the results of which show that (**f**) mDec-AAV-injected muscle contained significantly less fibrosis in the injured area than did the (**h**) control muscle at (**j**) 4 weeks after injury.

DISCUSSION

Results from these experiments show that decorin is able to activate the differentiation of skeletal muscle cells (C2C12) *in vitro* and enhances muscle regeneration in two mouse models *in vivo*. The mechanism behind decorin's accelerated muscle healing is not yet known; however, our results demonstrate that decorin up-regulates the expression of PGC-1α, follistatin, p21, and a variety of myogenic proteins (including MyoD) but down-regulates myostatin expression. These results, in addition to decorin's ability to neutralize the effects of TGF-β1, likely explain the beneficial action that decorin has on muscle cell differentiation and muscle regeneration.

Our previous studies have demonstrated that myogenic cells (including muscle-derived stem cells) in injured muscle can

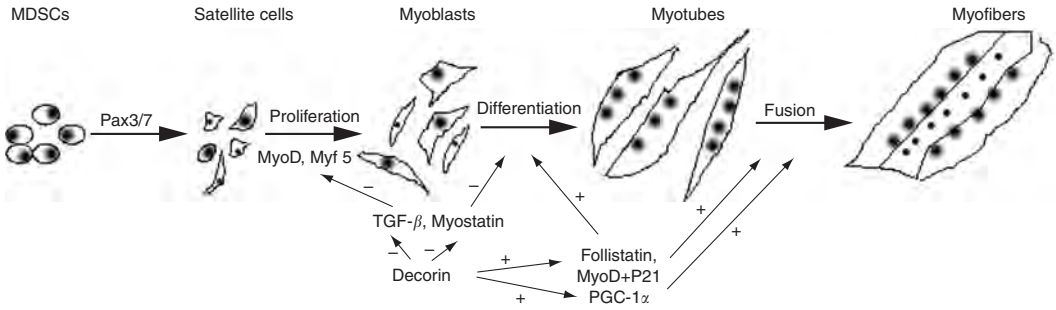

Figure 6 Schematic of the potential effect of decorin on muscle healing. Decorin may improve muscle healing through various pathways: inhibition of tumor growth factor (TGF)-β1, up-regulation of follistatin, peroxisome-proliferator-activated receptor-gamma co-activator-1α (PGC-1α), p21, and myogenic genes (such as *MyoD*), and down-regulation of myostatin expression. MDSCs, muscle-derived stem cells.

differentiate into fibrotic cells and that TGF-β1 is a major stimulator of this differentiation.[27,28] Using different animal models of muscle injury, we have investigated biological approaches to prevent fibrosis and thereby improve muscle healing.[11,27,29–31] We have used various molecules, such as decorin, that impede fibrosis by blocking TGF-β1 to facilitate the near-complete recovery of injured skeletal muscle.[11] The ability of decorin to inhibit TGF-β1 activity is the likely mechanism by which this molecule blocks fibrosis formation. However, our results indicate that the improved muscle healing observed after decorin treatment is due to both its inhibiting effect on fibrosis and its stimulating effect on muscle regeneration. **Figure 6** summarizes the potential effect of decorin on muscle healing.

The repair of injured skeletal muscle occurs through the activation of muscle precursor cells located between the basal lamina and the sarcolemma, including satellite cells and stem cells.[32] The activation and growth of these cells are regulated by various growth factors released by infiltrating lymphocytes, injured myofibers, and the extracellular matrix.[10,32] Some growth factors, such as insulin-like growth factor-1 and hepatocyte growth factor, can stimulate precursor cell proliferation and differentiation by increasing the transcriptional activity of the muscle basic helix–loop–helix.[33–36] Healing and organizational processes are dependent upon the extra- and intracellular signaling that induces the expression of myogenic genes, including *MyoD*, *Myf5*, and *myosin*.[37,38] When properly stimulated, precursor cells fuse with one another or with local myofibers to repair the damaged muscle.[39]

Muscle regeneration, the key event in muscle healing, is often incomplete, particularly in severely injured muscle.[10,11,27,28,32,40] The overgrowth of the extracellular matrix leads to significant local fibrosis (*i.e.*, fibrous scar formation) in the injured area, which can impede the formation of normal muscle fibers. The presence of fibrous scar tissue in injured muscle results in incomplete functional recovery and a propensity for re-injury.[41] Muscle regeneration and fibrosis in injured muscle often occur simultaneously and thus compete with one another during the muscle healing process.[32,36,40] A persistent imbalance between collagen biosynthesis and degradation contributes to hypertrophic scar formation and fibrosis in many tissues.[42,43] Several studies have revealed high levels of collagen in injured regions of skeletal muscle, and shown that inhibition of collagen deposition reduced the formation of scar tissue in injured skeletal muscle.[27,28,30,31]

Interactions between decorin and TGF-β1 have been observed in many tissues, and researchers have used various animal models to study the antifibrotic effect of decorin.[3,11,13,44] Researchers have also shown that hepatocyte growth factor can increase the level of decorin expression in fibroblasts, perhaps by activating the extracellular signal–regulated kinase 1/2 and p38 mitogen-activated protein kinase–mediated pathways.[14] Such findings could explain the antifibrotic effect of hepatocyte growth factor in a variety of tissues.[14,45–47] In endothelial cells, decorin binds with both insulin-like growth factor-I and its receptor to influence cell behavior.[17] Decorin can also control and suppress cancer growth and invasion, presumably by influencing the biological activity of growth factors such as TGF-β1, platelet-derived growth factor, vascular endothelial growth factor, and epidermal growth factor, all of which are released by cancer cells.[5,15] These decorin-induced effects appear to be mediated, at least in part, by a specific interaction between the decorin protein core and the epidermal growth factor receptor.[15,16] This interaction triggers a signal cascade that results in activation of mitogen-activated protein kinase, mobilization of intracellular calcium, up-regulation of p21, and, ultimately, the suppression of tumor growth.[18,19]

Cell cycle exit and the differentiation of muscle cells are coordinated by p21, which is essential for normal myogenic progenitor cell differentiation and skeletal muscle regeneration. Studies have indicated that p21 is necessary for MyoD-induced activity in cells, allowing them to enter into and be stabilized in a postmitotic state. Since MyoD plays a central role in the differentiation of muscle cells, TGF-β1 controls myostatin-related regulation of myogenesis in muscle cells by down-regulating both p21 and MyoD. In this study, we determined that the treatment of myoblasts with decorin down-regulated the expression of myostatin, which might influence p21 and myogenic protein expression. In addition, myostatin and follistatin interact directly in the skeletal muscle system. Follistatin can inhibit myostatin, leading to muscle differentiation in a concentration-dependent manner.[21] PGC-1α, which is expressed in several tissues, including brown fat and the skeletal muscle of mammals, activates mitochondrial biogenesis and oxidative metabolism.[26] PGC-1α is a principal factor involved in determining muscle fiber type in injured skeletal muscle and is involved in exercise-induced mitochondrial biogenesis.[48] In this experiment, we observed that decorin treatment increased PGC-1α expression in skeletal muscle cells. Combined with our previous research results, our current findings suggest that decorin not

only acts as an antifibrotic agent but also enhances muscle regeneration in skeletal muscle.

Successful muscle differentiation during limb development requires decorin expression.[6] Previous findings have shown that decorin can improve muscle healing by inhibiting fibrosis and that myoblasts and muscle satellite cells expressing decorin in an injured site regenerated damaged myofibers faster than the controls.[11,27] The results of this study demonstrate that decorin is also a potent stimulator of skeletal muscle regeneration. Myoblasts expressing decorin differentiated and fused to form myotubes and myofibers at a significantly higher rate than did normal myoblasts *in vitro* and *in vivo*. We attribute this enhanced differentiation to the up-regulation of p21, follistatin, PGC-1α, and myogenic gene expression and the down-regulation of TGF-β1 and myostatin. These results provide at least a partial explanation of the way in which decorin promotes muscle regeneration and may explain why there is such a high level of decorin expression in developing skeletal muscle. It is possible that decorin increases muscle fiber growth and limits the overgrowth of connective tissues. These findings indicate that decorin could be very useful in promoting the healing of muscles damaged by injury or disease.

MATERIALS AND METHODS

Gene transfection and transfer. An AAV-mDecorin plasmid, which encodes for a mouse decorin sequence under the control of the cytomegalovirus promoter (**Figure 1**), was used for gene transfection. This plasmid also contains a neomycin resistance gene to enable G418 selection. The AAV-mDecorin plasmid was transfected into 293 packaging cells and C2C12 cells with lipofectin; clone cells were selected for treatment of the cells with G418 (500 μg/mL) (Gibco BRL, Grand Island, NY) for 2 weeks. The selected decorin-transfected C2C12 clone cells (CD cells) were cultured in Dulbecco's modified Eagle's medium (Gibco BRL, Grand Island, NY) containing the same concentration of G418 for the remainder of the project.

The mDec-AAV vector was produced by co-transfection methods described previously by Dr. Xiao.[49] Muscle-derived stem cells and myoblasts (C2C12 cells) were each grown to 50–60% confluency. Fresh Dulbecco's modified Eagle's medium (without fetal bovine serum or penicillin/streptomycin) containing the mDec-AAV vector (5×10^4 particles/cell) was then added directly to the cells. The cultures were incubated at 37 °C in a 5% CO_2 incubator for 1 hour. Normal culture medium (Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and 1% Abs) was then added for another 24 hours, at which point the cells were collected for analysis of decorin expression by western blotting.

Differentiation of myoblasts and immunocytochemistry. Three different groups of cells (C2C12 cells, C2C12 cells cultured with decorin, and CD cells) were seeded into 12-well plates containing proliferation medium.[28] All cells were transferred into serum-free medium 12 hours later to induce differentiation. The myotubes that formed in the cultures were counted daily for 5 days, and the numbers were compared among the groups. We considered myotubes containing three or more nuclei to be large myotubes *in vitro*. At different time points, the cells were fixed with cold acetone (3 minutes) for immunostaining. Mouse anti-myosin heavy chain antibody (Novocastra Lab) at a 1:200 dilution was applied for 1 hour at room temperature (RT). The primary antibody was detected using anti-mouse-Cy3, 1:250 for 45 minutes at RT. Results were analyzed by fluorescent microscopy (Nikon microscope, Nikon, Melville, New York).

Real-time polymerase chain reaction. Total RNA was extracted from the treated and non-treated C2C12 cells using a Nucleospin column (Clontech, Mountan View, CA), and the complementary DNA was synthesized with SuperScript II reverse transcriptase (Invitrogen, Carlsbad, CA), both according to manufacturer's instructions. Primers specific for *myostatin*, *follistatin*, *p21*, and *PGC-1α* were designed using Oligo software (OligoPerfect Designer; Invitrogen, Carlsbad, CA). The protocol for amplification was as follows: 94 °C for 30 seconds, 58 °C for 30 seconds, and 72 °C for 30 seconds for 30 cycles. Polymerase chain reaction products were separated by size in a 1.5% agarose gel.

Western blot analysis. C2C12 and CD cells were lysed when cell density reached 70% confluency. The samples were separated on a 12% sodium dodecyl sulfate–polyacrylamide electrophoresis gel and transferred to nitrocellulose membranes used to perform immunostaining. The primary antibodies were anti-decorin (a gift from Dr. Fisher of the National Institutes of Health), anti-TGF-β1 (4 μg/mL; BD Pharmingen, San Diego, CA), anti-p21 (BD Pharmingen, San Diego, CA), anti-myf5, anti-myf6, anti-MyoD, and anti-myogenin (Santa Cruz Bio, Santa Cruz, CA), all at concentrations of 1:1,000, and anti-myostatin, anti-follistatin (Chemicon, Temecula, CA), and anti-desmin (Sigma, St. Louis, MO), all at concentrations of 1:2,000 for 1 hour at RT. Mouse anti-β-actin and anti-glyceraldehyde-3-phosphate dehydrogenase (Sigma, St. Louis, MO) were used for protein quantification and were diluted to 1:8,000. The secondary anti-rat horseradish peroxidase or anti-rabbit horseradish peroxidase (Pierce, Rockford, IL) was used at a concentration of 1:5,000 for 1 hour. Peroxidase activity was determined by enhanced chemiluminescence (Amersham Pharmacia Biotech, Piscataway, NJ), and the positive bands were detected on X-ray film. Northern Eclipse software v.6.0 (Empix Imaging, Mississauga, Canada) was used to evaluate all results.

Animal experiments. All animal experiments were approved by the Children's Hospital of Pittsburgh. The Animal Research Committee at the authors' institution approved all experimental protocols (No. 15/03).

Group 1: C2C12 and CD cell transplantation. Twenty-four female *MDX*/SCID mice (C57BL/10ScSn-*Dmd*^{mdx} crossed with C57BL/6J-*Prkdc*^{scid}/SzJ, 6–8 weeks of age) were used for the C2C12 and CD cell transplantation. C2C12 and CD clone cells were transduced with a retrovirus vector encoding for LacZ.[27] LacZ-positive CD cells (1×10^6) were injected into the left gastrocnemius muscles (GMs); the same quantity of LacZ-positive C2C12 cells were injected into the right GMs as a control. At various times after injection, mice were killed, and the GMs were collected for histological analysis by LacZ staining and immunohistochemistry to stain for dystrophin-positive myofibers.

Group 2: mDec-AAV gene therapy administered to injured skeletal muscle. Twenty mice (C57BL6J$^{+/+}$, 6 weeks old; Jackson Laboratory, Bar Harbor, ME) were used for these experiments. The mDec-AAV vector (2×10^{11} particles in 20 μL of Dulbecco's modified Eagle's medium) was injected directly into the left GM of each mouse; the contralateral leg was injected with the same volume of phosphate-buffered saline (20 μL) as a control. One week after injection, both GMs were lacerated in accordance with our previously described muscle injury model.[11,27,28,40] Mice were killed at different time points (5 days and 1, 2, 3, and 4 weeks after injury), and the GMs were collected for histological analysis by either hematoxylin and eosin or Masson's trichrome staining. The regeneration and fibrous scar tissue formation in the two groups were compared.

Immunohistochemical analysis. Serial 10-μm cryostat sections were prepared using standard techniques.[27,28] For immunohistochemistry, the slides were fixed with formalin (4%) for 5 minutes after LacZ staining, and then blocked with donkey serum (10%) for 1 hour. Rabbit anti-dystrophin antibody (Abcam, Cambridge, MA) was applied to the slides at a 1:300 dilution for 60 minutes at RT. The second antibody, goat anti-rabbit IgG (Alexa Fluor® 488; Molecular Probes, Eugene, OR), was used at a concentration of 1:200 for 45 minutes at RT. Negative controls were performed concurrently with all immunohistochemical staining. The nuclei of the sections were revealed using 4′,6′-diamidino-2-phenylindole hydrochloride

staining (Sigma, St. Louis, MO), and fluorescent microscopy was used to visualize the results as described above.

Statistical analysis. LacZ-positive myofibers were counted in 10 representative sections. Both the diameter and number of LacZ- and dystrophin-positive myofibers were assessed at different time points in each group. The statistical significance of differences between the various groups was determined using a *t*-test or one-way or two-way analysis of variance.

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Relationships between Transforming Growth Factor-β1, Myostatin, and Decorin

*IMPLICATIONS FOR SKELETAL MUSCLE FIBROSIS**

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Recent studies have shown that myostatin, first identified as a negative regulator of skeletal muscle growth, may also be involved in the formation of fibrosis within skeletal muscle. In this study, we further explored the potential role of myostatin in skeletal muscle fibrosis, as well as its interaction with both transforming growth factor-β1 and decorin. We discovered that myostatin stimulated fibroblast proliferation *in vitro* and induced its differentiation into myofibroblasts. We further found that transforming growth factor-β1 stimulated myostatin expression, and conversely, myostatin stimulated transforming growth factor-β1 secretion in C2C12 myoblasts. Decorin, a small leucine-rich proteoglycan, was found to neutralize the effects of myostatin in both fibroblasts and myoblasts. Moreover, decorin up-regulated the expression of follistatin, an antagonist of myostatin. The results of *in vivo* experiments showed that myostatin knock-out mice developed significantly less fibrosis and displayed better skeletal muscle regeneration when compared with wild-type mice at 2 and 4 weeks following gastrocnemius muscle laceration injury. In wild-type mice, we found that transforming growth factor-β1 and myostatin co-localize in myofibers in the early stages of injury. Recombinant myostatin protein stimulated myofibers to express transforming growth factor-β1 in skeletal muscles at early time points following injection. In summary, these findings define a fibrogenic property of myostatin and suggest the existence of co-regulatory relationships between transforming growth factor-β1, myostatin, and decorin.

Skeletal muscle injuries are one of the most common injuries encountered in sports, accounting for 10–55% of all sports related injuries (1–3). Despite their clinical significance, current treatments remain conservative, such as the RICE principle (rest, ice, compression, and elevation) and non-steroidal anti-inflammatory drugs. However, increasing evidence shows that the administration of non-steroidal anti-inflammatory drugs decreases regeneration and increases fibrosis by inhibiting inflammation (4–8). Although injured skeletal muscle can spontaneously undergo regeneration, muscle regeneration must compete with the ensuing formation of fibrosis, especially in acute injuries (9–11). The resulting excessive fibrotic tissue might form a dense mechanical barrier that prevents the regenerating muscle fibers from maturing (12, 13), thereby resulting in incomplete skeletal muscle healing (14, 15). Researchers have widely accepted that transforming growth factor-β1 (TGF-β1)[3] is a potent stimulator of fibrosis in various tissues (16–19) and is closely associated with skeletal muscle fibrosis as well (20). TGF-β1 levels are elevated in both dystrophic muscles and injured muscles (21, 22). Researchers have also shown that TGF-β1 effectively induces myofibroblastic differentiation of fibroblasts both *in vitro* and *in vivo* (23, 24). The resulting overgrowth of myofibroblasts is responsible for the ensuing excessive accumulation of fibrotic tissue (23, 24). We have previously reported that TGF-β1 plays a significant role in both the initiation of fibrosis and the induction of myofibroblastic differentiation of myogenic cells in injured skeletal muscle (20, 25). Additionally, we have shown that antifibrosis therapies, such as interferon-gamma (INF-γ), suramin, relaxin, and decorin (DCN), improve the healing of injured muscle both histologically and physiologically by blocking the activity of TGF-β1 (26–32). However, it is unclear whether TGF-β1 acts alone or requires interaction with other molecules during the development of muscle fibrosis. Indeed, recent studies have shown that

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[3] The abbreviations used are: TGF-β1, transforming growth factor-β1; MSTN, myostatin; INF, interferon; DCN, decorin; MSTN$^{-/-}$, myostatin knockout; MSTN$^{-/-}$/*mdx* mice, *mdx* mice with myostatin gene knockout; GM, gastrocnemius muscle; PM, proliferation medium; DM, differentiation medium; HS, horse serum; PP1 cells, a population of preplated cells; α-SMA, α-smooth muscle actin; FN, fibronectin; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; ELISA, enzyme-linked immunosorbent assay; PBS, phosphate-buffered saline; WT, wide-type; M.O.M., Mouse on Mouse; ECM, extracellular matrix; MRF, muscle regulatory factor; LTP, long-term proliferating; FLST, follistatin; Q-RT-PCR, quantitative reverse transcription-PCR; TβRII, TGF-β1 receptor type II.

myostatin (MSTN), a member of the TGF-β superfamily, may also be involved in fibrosis formation within skeletal muscle (33), although a direct link between MSTN and fibrosis has yet to be identified.

MSTN was initially identified as a negative regulator of muscle development (34), but unlike the ubiquitous expression of TGF-β1, MSTN is predominately expressed in skeletal muscle. MSTN knock-out ($MSTN^{-/-}$) mice, as well as cattle and humans with a naturally occurring MSTN gene mutation, are characterized by a dramatic and widespread increase in skeletal muscle mass (34–36). Interestingly, recent reports suggest that *mdx* mice (an animal model for Duchenne muscular dystrophy) in which expression of the MSTN gene has been ablated ($MSTN^{-/-}$/*mdx*) not only showed better skeletal muscle regeneration but also exhibited decreased fibrosis when compared with *mdx* mice ($MSTN^{+/+}$/*mdx*) (33). These results strongly suggest that MSTN plays an important role in muscle fibrosis. To investigate this possibility, we evaluated the effect of MSTN on fibrosis formation in injured skeletal muscle. Because TGF-β1 plays a major role in the formation of fibrosis, we hypothesized that a relationship between TGF-β1 and MSTN exists. Because DCN has been shown to strongly inhibit fibrosis formation in various tissues via blocking of TGF-β1 activity (26, 27, 37–40), we investigated the potential for DCN to inhibit the activity of MSTN as it does for TGF-β1. Our findings demonstrated that MSTN is involved with fibrosis formation and interacts with TGF-β1 and that DCN has the ability to counteract the action of MSTN. These results contribute to a better understanding of the mechanism of skeletal muscle healing and indicate that MSTN represents a potential pharmacological target for anti-fibrogenic therapy.

EXPERIMENTAL PROCEDURES

Isolation of Fibroblasts from Skeletal Muscle—The preplate technique was used to isolate fibroblasts from skeletal muscle (41). Collagen-coated flasks were used in the isolation process, because fibroblasts adhere more readily to collagen than myoblasts. After 6-week-old female C57BL/6J mice were sacrificed, their gastrocnemius muscles (GMs) were removed and minced into a coarse slurry. The muscle slurry was digested with 0.2% collagenase (type XI) for 1 h, followed by a dispase digestion (grade II, 240 ml) for 30 min, followed by a 0.1% trypsin digestion for a final 30 min at 37 °C. The extracted muscle cells were resuspended in proliferation medium (PM) consisting of Dulbecco's modified Eagle's medium (Invitrogen), 10% horse serum (HS, Invitrogen), 10% fetal bovine serum (Invitrogen), 1% penicillin/streptomycin (Invitrogen), and 0.5% chicken embryo extract (Accurate Chemical & Scientific Corp., Westbury, NY) and plated onto collagen-coated flasks. A population of preplated cells (PP1), consisting of mostly fibroblasts that attached within the first 2 h, was collected and used, in these experiments, as skeletal muscle-derived fibroblasts. This preplate technique was also used to isolate long-term proliferating (LTP) cells (muscle-derived stem cell-like cells) from WT and $MSTN^{-/-}$ muscle (41). Two hours after the initial plating, most of the rapidly adhering fibroblasts attached; the remaining nonadherent cells were transferred to a new collagen-coated flask every 24 h. As this process was repeated, the subsequent populations of late-adhering cells were identified as PP2, PP3, PP4, and PP5 in sequence. Following the collection of PP5, the rest of the cell suspension was incubated for an additional 72 h to allow the cells to attach in another collagen-coated flask. The final adherent cells are LTP cells (41).

Cell Culture—The NIH3T3 fibroblast cell line and the C2C12 myoblast cell line were purchased from the American Type Culture Collection (Manassas, VA). The cell lines or isolated PP1 fibroblasts were maintained in PM consisting of Dulbecco's modified Eagle's medium, 10% fetal bovine serum, and 1% penicillin/streptomycin until further needed. PP1 fibroblasts were plated onto collagen-coated 96-well plates for cell-proliferation analysis and onto 6-well plates for the evaluation of α-smooth muscle actin (α-SMA), fibronectin (FN), collagen (types Iα1, IIα2, and IIIα1), and MSTN expression. Following an overnight incubation, PM was replaced with serum-free medium supplemented with a serum replacement (Sigma) consisting of heat-treated bovine serum albumin, heat-treated bovine transferrin, and bovine insulin. This serum replacement does not contain growth factors, steroid hormones, glucocorticoids, or cell adhesion factors. We further supplemented this media with varying concentrations of recombinant human MSTN (Leinco Technologies, Inc., St. Louis, MO) for proliferation assays (0, 100, 500, or 1000 ng/ml) and for Western blot analysis (0, 100, or 500 ng/ml). After incubation for 48 h, an MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) cell proliferation assay kit (Roche Diagnostics, Germany) was used to measure cell proliferation ($n = 6$) following the instructions from the manufacturer. Western blot analysis was used to examine α-SMA, FN, and MSTN expression. Some of the above procedures were repeated using NIH3T3 fibroblasts to confirm the effect of MSTN on fibroblasts.

C2C12 myoblasts, a widely used myogenic cell line (42–44), were used to examine whether DCN neutralized the inhibitory effect of MSTN on cell differentiation. We seeded C2C12 myoblasts in 12-well plates in PM at a density of 10,000 cells/well. Following an overnight incubation, PM was replaced with fresh differentiation medium (DM) containing Dulbecco's modified Eagle's medium, 2% HS, and 1% penicillin/streptomycin. We maintained a total of four sets of cultured cells. The control set received only DM, whereas the other sets received DCN alone or 1 μg/ml MSTN combined with 0–50 μg/ml DCN ($n = 3$). Cells were cultured for 5 more days during which DM, MSTN, and DCN were changed every other day. Following a similar procedure, we examined whether recombinant follistatin (FLST) protein stimulated myogenic differentiation of C2C12 myoblasts ($n = 3$), and whether soluble TGF-β1 receptor type II (TβRII, 100 and 1000 ng/ml, R&D Systems, Inc., Minneapolis, MN) was able to attenuate MSTN-inhibited myoblast differentiation ($n = 3$).

Western Blot Analysis—After culturing, the cells were lysed with T-PER® Tissue Protein Extraction Reagent with the addition of protease inhibitors (Pierce). Equal amounts of cellular protein were loaded into each well and separated by 10% SDS-PAGE. Nitrocellulose membrane blotting was performed under standard conditions. The following primary antibodies were used for immunoblotting: mouse anti-β-actin IgG (1:8000, Sigma), mouse anti-glyceraldehyde-3-phosphate dehydrogen-

TABLE 1
Sequence of primer set

Gene name (GenBank™ no.)	Primer pair (S: sense primer, A: anti-sense primer)	PCR products
		bp
Procollagen type Iα1 (BC050014)	S: 5′-GAAGAACTGGACTGTCCCAAC-3′ A: 5′-CCTCGACTCCTACATCTTCTG-3′	103
Procollagen type Iα2 (AK075707)	S: 5′-TCTGGTAAAGAAGGCCCTGTG-3′ A: 5′-GTCCAGGGAATCCGATGTTG-3′	106
Procollagen type IIIα1 (AK041115)	S: 5′-AGGCTGAAGGAAACAGCAAA-3′ (45) A: 5′-TAGTCTCATTGCCTTGCGTG-3′	116
TGF-β1 (BC 013738)	S: 5′-CTAATGGTGGACCGCAACAAC-3′ A: 5′-CACTGCTTCCCGAATGTCTGA-3′	99
18 S rRNA (?)[a]		N/A

[a] Sequences of the primer pairs for 18 S rRNA not provided by Applied Biosystems Inc. for proprietary reasons.

ase IgG (1:5000, Abcam Inc., Cambridge, MA), rabbit anti-MSTN IgG (1:3000, Chemicon, Temecula, CA), mouse anti-α-SMA IgG (1:1000, Sigma), mouse anti-FN IgG (1:3000), and rat anti-TGF-β1 IgG (1:1000, BD Pharmingen, San Jose, CA).

Quantitative RT-RCR—Quantitative RT-PCR (Q-RT-PCR) was used to examine the mRNA expression levels of procollagen (types Iα1, Iα2, and IIIα1) in PP1 fibroblasts treated with MSTN (100, 200, and 500 ng/ml) for 12, 24, and 48 h. The mRNA was extracted using an RNeasy Plus kit (Qiagen). The cDNA templates for Q-RT-PCR were synthesized using a RETROscript® kit (Ambion Inc., Austin, TX). Q-RT-PCR was carried out in an ABI Prism 7000 sequence detector (Applied Biosystems Inc., Foster City, CA) with SYBR Green PCR Master Mix Reagent (Applied Biosystems) as a detector. All target gene expressions were normalized to 18 S rRNA levels. The primer pair of procollagen IIIα1 was from a previous study (45). The primer pairs are displayed in Table 1.

ELISA—Enzyme-linked immunosorbent assay (ELISA) was performed to determine whether recombinant MSTN protein stimulated TGF-β1 secretion in C2C12 myoblasts. C2C12 myoblasts were plated into a 48-well plate and exposed to a range of MSTN concentrations from 0 to 500 ng/ml. Fresh, recombinant MSTN protein was added every 2 days. Cell supernatants were collected at 2 and 4 days ($n = 5$). These supernatants were centrifuged to remove cell debris and stored at −80 °C until the ELISA was performed. The mouse/rat/porcine TGF-β1 immunoassay kit (R&D Systems, Inc.) was used to quantitatively measure the secreted TGF-β1 levels in cell culture supernatants, according to the manufacturer's protocol.

Immunocytochemistry—To monitor the differentiation capacity of the myogenic cells, they were fixed in cold methanol for 2 min after induction of differentiation in 12-well plates. Following a phosphate-buffered saline (PBS) wash, the cells were blocked with 10% HS (Vector Laboratories, Inc., Burlingame, CA) for 30 min, and then incubated with an anti-myosin heavy chain antibody (Sigma) in 2% HS overnight. A negative control was performed by omitting the primary antibody. The next day, after several PBS rinses, the cells were incubated with the secondary antibody goat anti-mouse IgG conjugated with Cy3 (Sigma) for 1 h. Hoechst 33258 dye was used in each experiment to stain cell nuclei. Fusion index (ratio of nuclei in myotubes to all nuclei) was calculated (%) to evaluate myogenic differentiation.

Animal Model—All experimental animal protocols were approved by the Animal Research and Care Committee at Children's Hospital of Pittsburgh (protocols 15-3 and 17-05). C57BL/6 wild-type (WT) (Jackson Laboratories, Bar Harbor, ME) and $MSTN^{-/-}$ mice (7–8 weeks of age) were used in this study. All $MSTN^{-/-}$ mice used were offspring of $MSTN^{-/-}$ homozygotes, and PCR was used to confirm the genotype of all $MSTN^{-/-}$ mice. The RT-PCR test was randomly used to confirm the lack of MSTN gene transcription in $MSTN^{-/-}$ mice throughout the experiments. The skeletal muscle mass of $MSTN^{-/-}$ mice and WT mice were also compared to confirm the desired phenotype. The mice were anesthetized with isoflurane controlled under an IMPAC6 anesthetic delivery machine (VetEquip, Pleasanton, CA). Both GMs of each mouse were laterally lacerated to create an injury model as previously described (27–29). A surgical blade (no. 11) was used to make a lateral laceration through 50% of the muscle width and 100% of the muscle thickness in the area of the GM with the largest diameter. We harvested the mouse GMs at 2 and 4 weeks post-surgery. There were 6–8 mice (12–16 GMs) in the WT and $MSTN^{-/-}$ mouse groups for both time points. The muscles were isolated, removed, and snap-frozen in 2-methylbutane pre-cooled in liquid nitrogen. After Masson's trichrome staining (IMEB Inc., Chicago, IL), Northern Eclipse software (Empix Imaging, Inc., Cheektawaga, NY) was used to measure areas of fibrotic tissue in the injured sites. In each sample, three representative non-adjacent sections were chosen. The ratio of the fibrotic area to the cross-sectional area was used to estimate the extent of fibrosis formation. To determine the skeletal muscle's regeneration efficiency, minor axis diameters (the smallest diameter) of regenerating muscle fibers were measured using Northern Eclipse software on cross-sections of GMs. The diameters of over 350 consecutively centro-nucleated myofibers were measured in each GM.

To analyze the expression of MSTN in the injured GM, 18 8-week-old female C57BL/6 WT mice underwent bilateral GM laceration. Mice were sacrificed at 1, 3, 5, 7, 10, 14, 21, and 30 days after injury ($n = 3$ for each time point), and GMs were harvested, frozen, and stored at −80 °C.

300,000 LTP cells obtained from $MSTN^{-/-}$ mice were transplanted in the GMs of 3 8-week-old *mdx*/scid mice using a protocol previously described (41). The same amount of cells obtained from WT mice was injected into contralateral GMs of *mdx*/scid mice to serve as our control. Mice were sacrificed after 4 weeks, and GMs were frozen in liquid nitrogen. Immunostaining with anti-mouse dystrophin antibody (Abcam Inc.) was performed to detect dystrophin-positive myofibers that regenerated from transplanted cells.

To examine whether the injection of MSTN induced TGF-β1 expression, we injected MSTN (1000 ng in 10 μl of PBS) into the non-injured GM of WT mice. Contralateral GMs were injected with 10 μl of PBS and served as a control. Three WT mice were used at each time point. Mice injected with MSTN were sacrificed at 4, 10, 24, and 48 h after injection ($n = 3$ for each time point). Immunohistochemical staining was performed to detect MSTN and TGF-β1 expression in muscle fibers.

Immunohistochemistry—Frozen GMs were sectioned at 10-μm thickness, and immunohistochemical analysis was performed to detect MSTN and TGF-β1 expression. Tissue sections were fixed in 4% formalin for 5 min followed by two 10-min washes with PBS. The sections were then blocked with 10% HS for 1 h. The rabbit MSTN primary antibody was diluted 1:100 in 2% HS and incubated with sections overnight at 4 °C. The following day, the sections were washed three times with PBS and then incubated with the secondary antibody, goat anti-rabbit IgG conjugated with Cy3 (Sigma). The Mouse-on-Mouse immunodetection kit (M.O.M., Vector Laboratories, Inc.) was then used to stain for TGF-β1 following the manufacturer's protocol. The slides were incubated with M.O.M. blocking reagent for 1 h, washed with PBS, and then incubated with M.O.M. diluent for 5 min. TGF-β1-specific primary antibodies (Vector Laboratories, Inc.) were diluted 1:150 in the M.O.M. diluent and incubated with the slides for 30 min. After washing with PBS, the sections were incubated with anti-mouse IgG conjugated with fluorescein isothiocyanate (diluted 1:200 with M.O.M. diluent, Sigma) for 1 h. Hoechst 33258 dye was used to stain the nuclei. In a separate experiment following a similar procedure, polyclonal rabbit anti-DCN IgG (LF-113, National Institute of Dental Research, Bethesda, MD) was used to stain tissue sections of WT and $MSTN^{-/-}$ GMs 2 weeks after laceration.

Statistical Analysis—All of the results from this study are expressed as the mean ± S.D. The differences between means were considered statistically significant if $p < 0.05$. The Student's *t* test was used to compare the difference in skeletal muscle regeneration, fibrosis formation between $MSTN^{-/-}$ and WT mice, and the myogenic differentiation capacity between $MSTN^{-/-}$ and WT LTP cells. All other data were analyzed by analysis of variance followed by post hoc Tukey's multiple comparison test. *Error bars* on the figures represent the ±S.D. (*, $p < 0.05$; **, $p < 0.01$).

RESULTS

Effects of MSTN on Fibroblasts—MTT proliferation tests showed that, after 48 h of incubation, MSTN significantly stimulated the proliferation of PP1 and NIH3T3 fibroblasts in a dose-dependent manner (Fig. 1*A*). α-SMA, the actin isoform originally found in contractile vascular smooth muscle cells, has been the most reliable marker of myofibroblasts to date (24). Western blot analysis indicated that MSTN (100 and 200 ng/ml) increased α-SMA expression in PP1 and NIH3T3 fibroblasts (Fig. 1*B*). Q-RT-PCR revealed that MSTN stimulated procollagen (type Iα1, Iα2, and IIIα1) mRNA expression at 48 h (Fig. 1*C*). Additionally, MSTN stimulated the expression of FN protein, a component of the extracellular matrix (ECM), in PP1 fibroblasts (Fig. 1*D*).

MSTN Expression in Injured Skeletal Muscle—After laceration injury, different time points were selected to detect MSTN expression in GMs. The degenerative and repair remodeling phases were represented by post-injury time points of 1–3 and 5–30 days following injury, respectively. Immunostaining for MSTN indicated MSTN expression within degenerative myofibers at 1 and 3 days after the injury (data not shown). On day 5, by the time a majority of newly regenerating myofibers was seen, faint MSTN signals were detected in the cytoplasm of regenerating centro-nucleated myofibers (*red fluorescence* and *white arrowheads*), whereas green collagen IV immunostaining indicates basal lamina of myofibers (Fig. 2*A*). MSTN expression was also observed in the nuclei of both the mononuclear cells (*white arrows*) and the regenerating centro-nucleated myofibers (Fig. 2*A*), which is especially obvious in the enlarged image (*white arrowhead* Fig. 2*A*, *inset*). On day 7 (Fig. 2*B*), a decrease in MSTN expression within most of the regenerating myofiber cytoplasm was seen (*white arrowheads*), whereas some myotubes without intact basal lamina were strongly stained with MSTN antibody, which is increased 14 days post-injury (*white arrows*, Fig. 2*C*). The nuclei of myofibers remained MSTN-positive (*yellow arrowhead*, Fig. 2, *B* and *C*, *insets*). MSTN staining disappeared from most regenerated myofibers 30 days after laceration (*white arrowheads*, Fig. 2*D*). Fig. 2, *E*, *F*, *G*, and *H*, depict negative controls of injured muscle at 5, 7, 14, and 30 days after laceration, respectively, where the MSTN antibody was replaced by the non-immune rabbit IgG. Collagen type IV was also stained on these samples to visualize the basal lamina.

Reduced Fibrosis and Enhanced Skeletal Muscle Regeneration in $MSTN^{-/-}$ Mice after Laceration—At 2 weeks following injury, we observed extensive deposition of collagenous tissue in the WT and $MSTN^{-/-}$ mice (data not shown). After 4 weeks, the deepest area of the injured site was filled with regenerating myofibers of large diameter, and the fibrotic region was limited to the superficial zone of the laceration site (Fig. 3*A*). We observed fewer fibrotic connective tissue deposits between regenerating myofibers in the injured muscle of $MSTN^{-/-}$ mice compared with the prominent scar region in the injured WT mouse muscle (Fig. 3*A*). Quantification of fibrotic tissue (*i.e.* the ratio of the fibrotic area to the cross-sectional area) revealed that there was a significantly smaller fibrous area in $MSTN^{-/-}$ skeletal muscle as compared with WT skeletal muscle at 2 weeks (11.5 ± 3.5% *versus* 15.3 ± 3.1%; $p < 0.01$) and at 4 weeks (2.1 ± 0.4 *versus* 6.3 ± 2.1; $p < 0.01$) after injury (Fig. 3*B*).

We used the minor axis diameter (smallest diameter) of centro-nucleated regenerating myofibers to evaluate skeletal muscle regeneration after laceration injury. At 2 weeks after GM laceration, regenerating myofibers were relatively small (data not shown). At 4 weeks, some large, mature myofibers could be observed among the small, centro-nucleated, regenerating myofibers (Fig. 3*C*). Quantification showed that $MSTN^{-/-}$-regenerating myofibers had diameters 38.8% larger than WT myofibers (36.1 ± 2.5 μm *versus* 26.0 ± 2.2 μm, $p < 0.01$) at 2 weeks after laceration, and the mean diameter of regenerating myofibers in $MSTN^{-/-}$ mice remained 21.1% larger than the

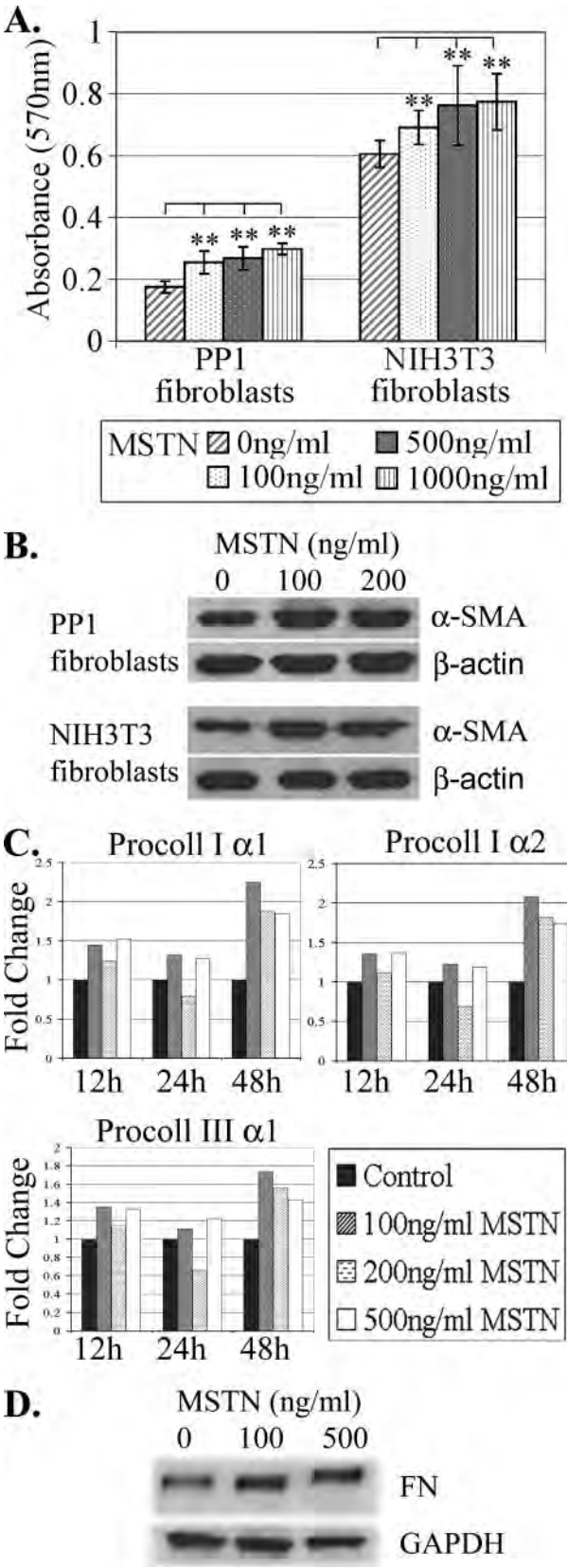

FIGURE 1. **MSTN stimulated fibroblast proliferation and fibrotic protein expression in fibroblasts.** *A*, both muscle-derived fibroblasts (*PP1*) and NIH3T3 fibroblasts were cultured with MSTN, varying in concentration from 0 to 1000 ng/ml for 48 h. Cell proliferation was determined by MTT assay. These results are presented as absorbance values ($n = 6$) of purple formazan crystal at 570 nm, which directly correlates to the number of living cells. Fibroblasts were cultured in DM for 2 days with the addition of various concentrations of MSTN. Expressions of different proteins were analyzed by Western blot. *B*, the expression of α-SMA in PP1 fibroblasts or NIH3T3 fibroblast is shown. *C*, Q-RT-PCR analysis of procollagen (types Iα1, Iα2, and IIIα1) mRNA expression in PP1 fibroblasts treated with MSTN. Results are presented as the ratio against the gene expression in the control. *D*, expression of FN in PP1 fibroblasts after MSTN treatment (*, $p < 0.05$; **, $p < 0.01$).

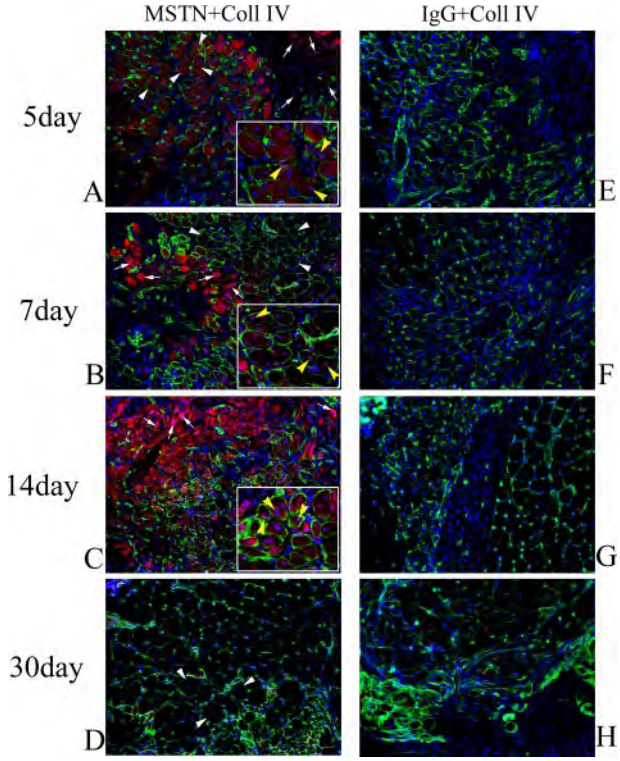

FIGURE 2. **MSTN localization in injured GMs.** *A–H*, GMs from WT mice were harvested at different time points after laceration injury, and frozen sections were immunostained with rabbit anti-MSTN and goat anti-collagen type IV antibodies. MSTN, collagen IV, and cell nuclei are *red*, *green*, and *blue*, respectively (*A–D*). Non-immune rabbit IgG was used as negative control for rabbit anti-MSTN antibody (*E–H*), but were stained with the collagen type IV antibody. *A*, at 5 days, faint MSTN signals could be detected in the cytoplasm of newly formed myofibers (*white arrowheads*) with basal lamina, and a relatively higher MSTN staining can be observed in the nuclei of regenerating myofiber (*yellow arrowhead*) and mononuclear cells (*white arrow*). *B*, at 7 days, MSTN staining is not evident in the cytoplasm of most regenerating myofibers (*white arrowhead*), whereas some of the regenerating small myotubes without basal lamina show intense MSTN staining in the cytoplasm (*white arrows*). *Yellow arrowheads* in the *inset* indicate positive signal in nuclei of regenerating myofibers. *C*, at 14 days, there were more MSTN-positive myotubes without basal lamina (*white arrow*). *Yellow arrowheads* in the *inset* indicate positive signal in nuclei of regenerating myofibers. *D*, at 30 days, most of regenerating myofibers were MSTN-negative (*arrowheads*). (Magnification, ×200; *inset* magnification, ×400.)

mean diameter of regenerating myofibers in the WT mice (37.7 ± 2.7 μm *versus* 31.1 ± 1.8 μm, $p < 0.01$) 4 weeks after injury (Fig. 3*D*). The distribution of the regenerating myofiber diameters showed that there was an increase in the percentage of larger regenerating myofibers in MSTN$^{-/-}$ mice compared with WT mice (*e.g.* ~7.38% of regenerating myofiber diameters in MSTN$^{-/-}$ mice fell into a range of 50–55 μm *versus* 1.92% of those in WT mice).

Improved Myogenic Potential with MSTN$^{-/-}$ LTP Cells—LTP cells were isolated from WT and MSTN$^{-/-}$ mice. When we cultivated these MSTN$^{-/-}$ LTP cells in low serum medium, they differentiated into myotubes that were significantly larger

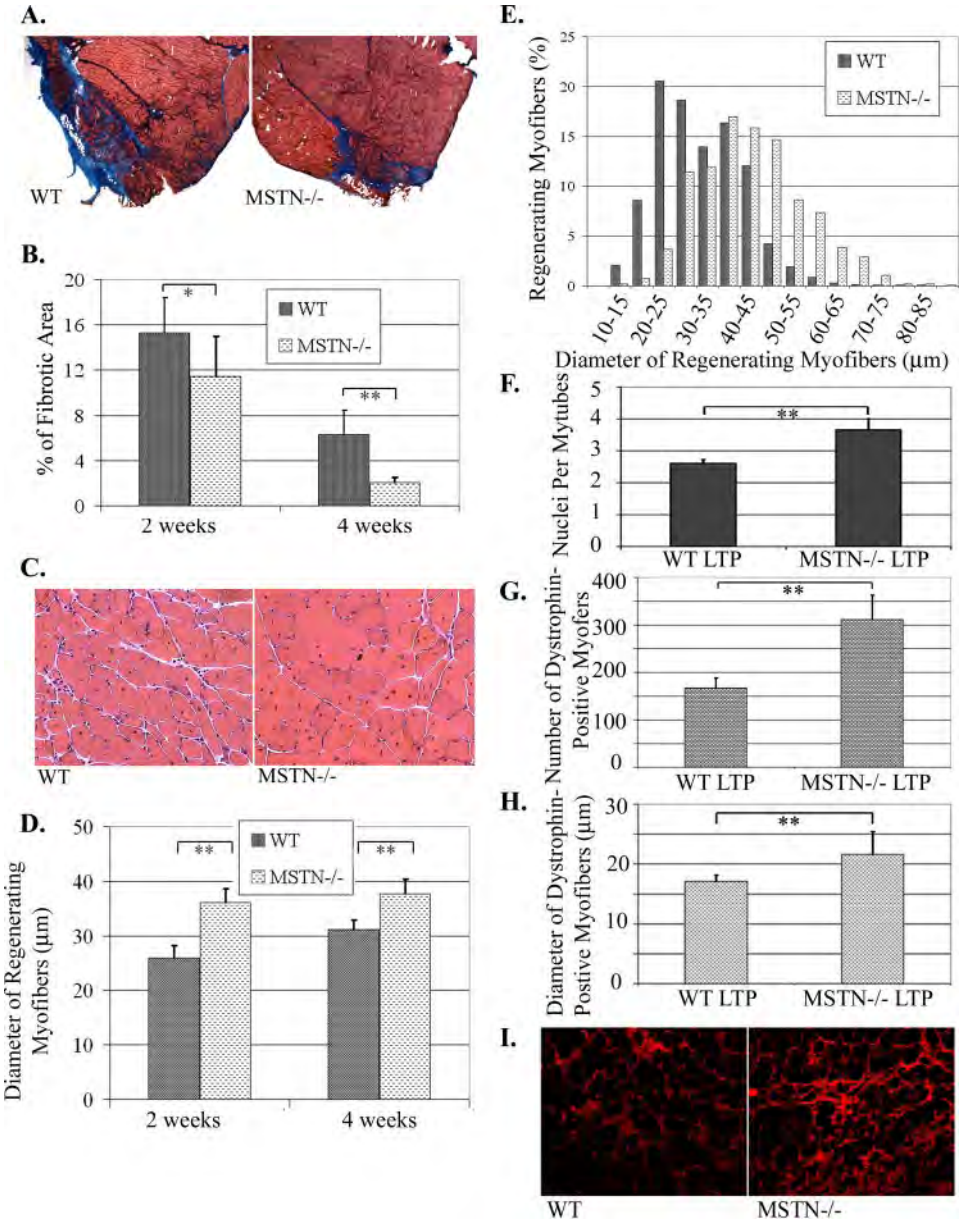

FIGURE 3. **Inhibition of MSTN favors skeletal muscle regeneration.** *A*, sections from injured WT and MSTN$^{-/-}$ GMs were stained with Masson's trichrome-staining protocol 4 weeks after laceration to determine fibrotic tissue levels. As a result, collagenous tissue is stained *blue*. *B*, quantification of fibrotic tissue of WT *versus* MSTN$^{-/-}$ GMs 2 and 4 weeks after laceration. *C*, myofibers in WT and MSTN$^{-/-}$ GMs were visualized by hematoxylin and eosin staining 4 weeks after laceration. Regenerating myofibers were distinguished by their centralized nuclei. *D*, quantification of the diameters of regenerating myofibers. *E*, the distribution of regenerating myofiber diameters at 4 weeks after laceration injury. *F*, myogenic differentiation capacity of WT and MSTN$^{-/-}$ LTP cell *in vitro*. *In vivo*, transplantation of MSTN$^{-/-}$ LTP into *mdx*/scid mice led to a high number of dystrophin-positive muscle fibers when compared with WT LTP. *G*, the number of dystrophin-positive myofibers was counted. *H*, the diameter of dystrophin-positive myofibers was measured. *I*, increased DCN immunostaining in injured skeletal muscle of MSTN$^{-/-}$ mice compared with WT mice 2 weeks after laceration. DCN (*red*) is detected in the ECM between myofibers. (Magnifications: in *C* and *I*, ×200; in *A*, ×100; *, $p < 0.05$; **, $p < 0.01$.)

(more nuclei per myotube, $n = 3$) than the myotubes formed by the fusion of WT LTP cells (Fig. 3*F*). When we injected the MSTN$^{-/-}$ LTP cells into the muscle of *mdx*/scid mice, they regenerated significantly more dystrophin-positive muscle fibers than did the WT LTP (Fig. 3*G*). These regenerating muscle fibers were also significantly larger in diameter (Fig. 3*H*).

Elevated DCN Expression in Injured MSTN$^{-/-}$ Mice—To investigate the underlying mechanism for improved muscle healing in MSTN$^{-/-}$ mice, we examined the expression of DCN, a molecule that has been shown to decrease fibrosis and enhance muscle regeneration (20, 27) in injured MSTN$^{-/-}$ skeletal muscle. Immunohistochemical staining revealed that there was more abundant DCN expression in the regenerating skeletal muscle of MSTN$^{-/-}$ mice than that of WT mice 2 weeks after injury (Fig. 3*I*). This higher level of DCN expression may be involved with the increased regeneration and decreased fibrosis observed in the injured muscle of MSTN$^{-/-}$ mice.

Relationship between TGF-β1 and MSTN$^{-/-}$—Western blot analysis showed that the levels of MSTN in C2C12 myoblasts treated with different concentrations of TGF-β1 were elevated in a dose-dependent manner when compared with non-treated controls, suggesting that TGF-β1 stimulates MSTN expression in C2C12 myoblasts (Fig. 4*A*). After incubation with increasing concentrations of recombinant MSTN protein, MSTN was shown to stimulate TGF-β1 expression in C2C12 myoblasts (especially with the highest dose) at 4 days post-stimulation (Fig. 4*B*). Furthermore, ELISA showed that MSTN significantly increased TGF-β1 secretion by C2C12 myoblasts in a dose-dependent manner at 2 and 4 days. After 4 days of stimulation with MSTN (500 ng/ml), C2C12 myoblasts secreted ~2-fold more TGF-β1 as compared with control cells (Fig. 4*C*). Q-RT-PCR revealed that MSTN (100, 200, and 500 ng/ml) also increased TGF-β1 mRNA expression 48 h post-stimulation (Fig. 4*D*).

PP1 fibroblasts did not express detectable MSTN protein. However, after treatment with MSTN (100 and 200 ng/ml) for 48 h, PP1 fibroblasts began to express MSTN as indicated by Western blot analysis (Fig. 4*E*). MSTN also stimulated MSTN expression in C2C12 myoblasts (Fig. 4*E*). MSTN-induced MSTN autocrine expression in PP1 fibroblasts is reduced by soluble TβRII, which blocks the TGF-β1 signaling pathway (Fig. 4*F*). Moreover, our results indicated that soluble TβRII was also able to restore MSTN-inhibited C2C12 myoblast differentiation (Fig. 4*G*). We also examined whether exogenous

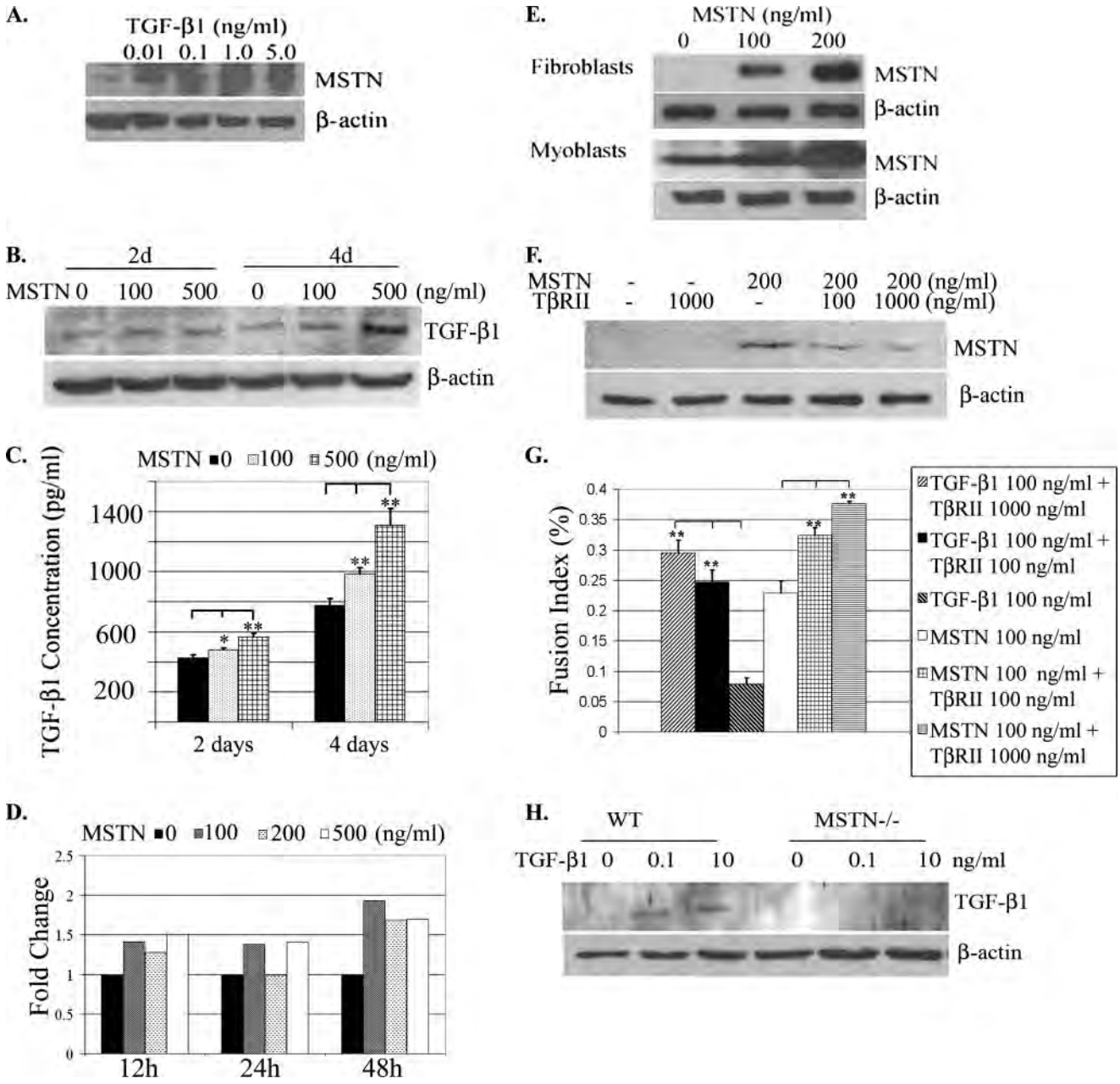

FIGURE 4. **The relationship between TGF-β1 and MSTN *in vitro*.** *A*, Western blot analysis of MSTN expression in C2C12 myoblasts treated with different concentrations of TGF-β1 ranging from 0 to 5.0 ng/ml for 48 h. *B*, C2C12 myoblasts were treated with different concentrations of MSTN in DM. Cell lysates were collected at 2 and 4 days to examine TGF-β1 expression by Western blot; *C*, while the conditioned medium was collected at the same time points, the levels of TGF-β1 in the medium were also analyzed by ELISA. *D*, Q-RT-PCR for TGF-β1 after MSTN treatment (100, 200, and 500 ng/ml) in PP1 fibroblasts. *E*, the level of MSTN expression in PP1 fibroblasts *and C2C12 myoblasts* treated with MSTN recombinant protein. *F*, Western blots were used to determine MSTN expression level in PP1 fibroblasts after cells were treated with either MSTN or both MSTN and soluble TβRII for 48 h. *G*, C2C12 myoblasts were cultured in DM with different treatments, TGF-β1, MSTN, TGF-β1 and TβRII, or MSTN and TβRII, for 4 days. Fusion indexes were used to access impacts of treatments on C2C12 myoblast differentiation. *H*, myoblasts isolated from WT, and MSTN$^{-/-}$ GMs were grown for 48 h under stimulation by TGF-β1. Western blot analysis was used to detect TGF-β1 expression in WT and MSTN $^{-/-}$ cells (*, $p < 0.05$; **, $p < 0.01$).

TGF-β1 recombinant protein was able to stimulate autocrine expression of TGF-β1 in MSTN$^{-/-}$ muscle cells as it does in C2C12 myoblasts (20). We observed that exogenous TGF-β1 could induce its autocrine expression in WT primary myoblasts but not on primary MSTN$^{-/-}$ myoblasts (Fig. 4*H*).

In vivo, We observed co-expression of TGF-β1 (*green*) and MSTN (*red*) in degenerative myofibers 1 and 3 days after laceration injury (*white arrow*, Fig. 5*A*). By day 5, MSTN was detected mainly in the nuclei of the regenerating myofibers (*white arrowhead*) with the exception of a few MSTN-positive necrotic myofibers, whereas TGF-β1 was present in the surrounding ECM (*white arrow*). MSTN was still detected in the nuclei of regenerating myofibers 21 days after injury (*white arrow*, Fig. 5*A*). The injection of MSTN into non-injured GMs induced TGF-β1 expression in the myofibers at 4, 10, and 24 h after injection. As shown in Fig. 5*B*, MSTN (*red*) and TGF-β1 (*green*) were co-expressed in myofibers at

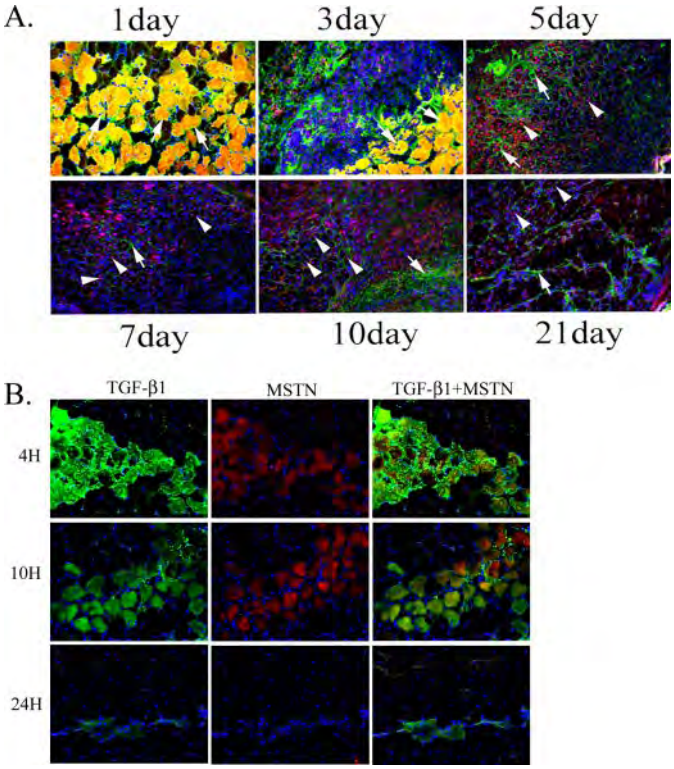

FIGURE 5. **The relationship between TGF-β1 and MSTN *in vivo*.** *A*, both GMs of each adult WT mouse underwent laceration injury. Mice GMs were harvested at the indicated times. Double staining of TGF-β1 (*green*) and MSTN (*red*) was performed. In the 1- and 3-day images, a *white arrow* indicates degenerative myofibers. At all other time points, the *white arrow* and *arrowhead* indicate ECM and nuclei of myofibers, respectively. *B*, co-localization of TGF-β1 and MSTN in myofibers after recombinant MSTN protein injection. We injected 1000 ng of MSTN protein in 10 μl of PBS into GMs of WT mice. Mice were sacrificed at different time points after injection. Frozen sections of GMs were double-stained with anti-TGF-β1 and anti-MSTN antibodies (magnification, ×200).

4 and 10 h. After 24 h, MSTN disappeared, and only a few TGF-β1-positive myofibers could be observed.

DCN Counteracts the Effect of MSTN—As previously shown in Fig. 1*A*, 0.1 μg/ml MSTN significantly stimulated PP1 fibroblast proliferation. This dosage was selected to examine whether DCN could reduce the proliferative influence of MSTN on PP1 fibroblasts. After PP1 fibroblasts were incubated with MSTN and exposed to varying concentrations of DCN for 48 h, MTT assay revealed that the addition of DCN significantly repressed the stimulatory effect of MSTN on PP1 proliferation in a dose-dependent manner as expected (Fig. 6*A*). These findings are comparable to a previous report showing that DCN blocked the stimulatory effect of TGF-β1 on PP1 fibroblasts (27).

Our earlier results indicated that MSTN induced its own expression, in an autocrine manner, in PP1 fibroblasts (Fig. 4*E*). Therefore, we examined the ability of DCN to block the MSTN autocrine expression in PP1 fibroblasts. As previously shown, PP1 fibroblasts that were not treated with MSTN failed to express detectable MSTN protein, whereas PP1 fibroblasts treated with MSTN showed a high level of MSTN expression in comparison to the control (Figs. 4*E* and 6*B*). However, DCN decreased MSTN autocrine expression by PP1 fibroblasts in a dose-dependent manner (Fig. 6*B*).

Our previous experiments showed that 1 μg/ml MSTN almost completely inhibited myoblast differentiation (data not shown). Therefore, we chose this dose to assess whether DCN treatment could reverse MSTN-inhibited myogenic differentiation in C2C12 cells. Except for the control cells, the cultures were treated with DCN alone or 1 μg/ml MSTN combined with increasing concentrations of DCN (0–50 μg/ml). Following a 5-day incubation, DCN-treated groups (data not shown) and controls showed widespread myosin heavy chain-positive myotubes, whereas cells treated with MSTN alone contained only a few myotubes (Fig. 6*C*). The addition of DCN reversed the inhibition of MSTN on myogenic differentiation, as indicated by the increase in the number and size of myotubes in comparison to the MSTN-treated group (Fig. 6*C*). Measurements showed that DCN treatment promoted C2C12 myoblast differentiation by significantly increasing fusion indexes in a dose-dependent manner (Fig. 6*D*), suggesting that DCN attenuated the inhibitory effect of MSTN and, thereby, stimulated myoblast fusion.

Inhibitory Effects of DCN on MSN May Be Mediated by FLST—To further explore whether DCN regulated MSTN activity via an intermediate molecule, we investigated the effect of DCN on the expression of FLST, which is able to bind to MSTN and suppress its activity (46). We found an up-regulation of FLST expression by C2C12 myoblasts 48 and 72 h after addition of 10 μg/ml DCN (Fig. 7*A*). Our results also revealed the ability of FLST to stimulate myogenic differentiation, which was demonstrated by the presence of larger myotubes containing more nuclei in comparison to the control group (Fig. 7*B*). In a dose-dependent manner, FLST treatment led to a significant increase in fusion index (Fig. 7*C*) compared with the control group, suggesting that FLST promotes myogenic differentiation and accelerates the maturation of myotubes.

DISCUSSION

MSTN has been drawing more and more attention due to mounting evidence indicating that inhibition of MSTN significantly improves skeletal muscle diseases such as muscle dystrophy. But, the role of MSTN in injured skeletal muscle and its relationships with other molecules such as TGF-β1 and DCN (important key factors in muscle healing) remain unknown. Recent studies reported by Yamanouchi *et al.* (47) highlight the expression of MSTN in fibroblasts in injured skeletal muscle, suggesting that fibroblasts may be a source of MSTN. Previously, we have shown that TGF-β1 significantly promotes proliferation of PP1 fibroblasts (27). Here, our *in vitro* study shows that MSTN activates fibroblasts by stimulating fibroblast proliferation and inducing their expression of α-SMA analogous to that of TGF-β1. Like TGF-β1 (48), MSTN may transiently attract fibroblasts into an injury site, further inducing them to express MSTN in an autocrine fashion; they then differentiate into myofibroblasts, thereby accelerating the deposition of the ECM. Researchers widely believe that prolonged presence and excessive activity of myofibroblasts is associated with the abnormal accumulation of ECM components in injured and diseased tissue (49, 50). Moreover, MSTN has been shown to induce procollagen (types Iα1, Iα2, and IIIα1), mRNA, and FN protein expression in PP1 fibroblasts. McCroskery *et al.* (51) recently confirmed the correlation of MSTN expression to the

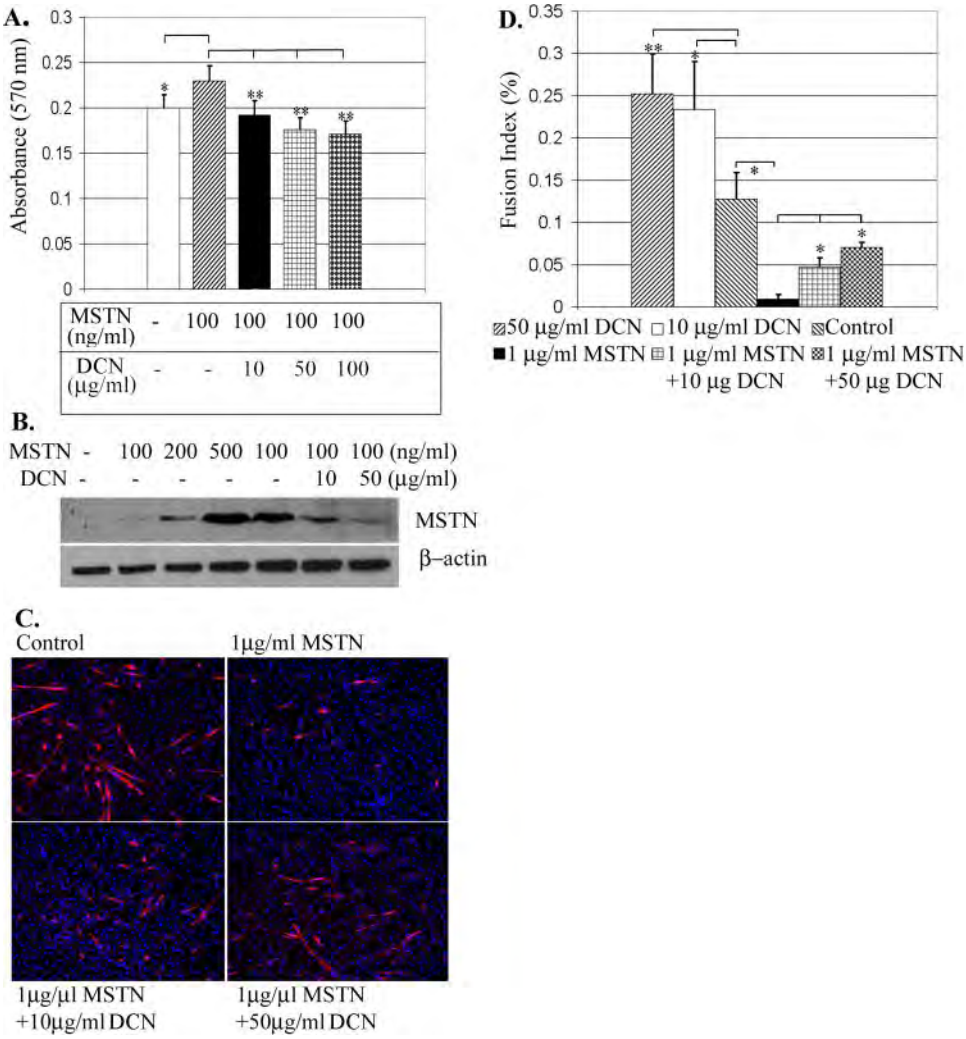

FIGURE 6. **DCN blocks the effects of MSTN on PP1 fibroblasts and C2C12 myoblasts.** *A*, PP1 fibroblasts were treated for 48 h with 100 ng/ml MSTN or combinations of MSTN and DCN. Non-treated cell cultures were used as a control. MTT assay was performed to assess cell proliferation. *B*, after incubation of PP1 fibroblasts with MSTN, or a combination of MSTN and DCN, Western blot analysis was performed to determine whether DCN reduced the autocrine expression of MSTN in PP1 fibroblasts stimulated with MSTN. *C*, C2C12 myoblasts were cultured without treatment, with 1 μg/ml MSTN alone, or co-incubated with 1 μg/ml MSTN and different concentrations of DCN for 5 days. Myotubes were monitored by anti-skeletal myosin heavy chain immunostaining; nuclei were stained by Hoechst 33258 (magnification, ×100). *D*, fusion indexes were determined to estimate the differentiation capacity of C2C12 myoblasts in response to different treatments.

formation of fibrosis by showing less fibrosis formation in the notexin-damaged tibialis anterior muscle in MSTN$^{-/-}$ mice 4 weeks after injury as compared with WT mice. Given the results collected in our *in vitro* study, we hypothesized that a lack of MSTN in knock-out mice would decrease the proliferation of fibroblasts and reduce their production of collagenous tissue in injured skeletal muscle. This was made evident by a significant decrease in the formation of fibrosis in MSTN$^{-/-}$ mice at 2 and 4 weeks after injury when compared with WT mice. Moreover, we found an elevated expression level of DCN, an inhibitor of TGF-β1, in injured MSTN$^{-/-}$ skeletal muscles compared with injured WT muscles at 2 weeks after injury. In accordance with this result, increased DCN mRNA has been observed in regenerating MSTN$^{-/-}$ muscle (51). Increased DCN might inhibit the effect of TGF-β1, thereby partially explaining the reduced fibrosis and enhanced regeneration in injured MSTN$^{-/-}$ muscle. To understand the mechanism by which MSTN$^{-/-}$ muscle displays less fibrosis than WT muscle after injury, the expression levels of TGF-β1 in injured WT mice *versus* that expressed in injured MSTN$^{-/-}$ mice should be compared more closely.

As members of the TGF-β superfamily, TGF-β1 and MSTN share many similarities in structure, signaling pathway, and function (52, 53). It has also been shown that TGF-β1 plays a critical role in skeletal muscle fibrosis after injury (20, 26–32). Because both TGF-β1 and MSTN promote fibrosis, it is very important to understand the potential relationships between these two molecules. Recent reports demonstrated that exogenous TGF-β1 strongly stimulated the expression of MSTN in C2C12 myoblasts (44). In fact, our *in vitro* data show that TGF-β1 increases MSTN expression in C2C12 myoblasts (and *vice versa*), and TGF-β1 and MSTN are found to co-localize in the same myofibers shortly after MSTN injection or after injury.

We found that MSTN is able to induce its autocrine expression in both fibroblasts and myoblasts. In the presence of soluble TβRII, MSTN autocrine expression in fibroblasts is decreased. We have known that MSTN inhibits C2C12 myoblast differentiation. When TβRIIs are blocked by soluble TβRII, the ability of MSTN to inhibit C2C12 myoblast differentiation is reduced. Apart from that, Q-RT-PCR results show that MSTN also stimulates TGF-β1 mRNA expression in PP1 fibroblasts. Our previous study has shown that TGF-β1 is able to induce autocrine expression of TGF-β1 in C2C12 myoblasts (20), nevertheless, our present data revealed that TGF-β1 failed to induce its autocrine expression in MSTN$^{-/-}$ primary muscle cells. Although TGF-β1 and MSTN may target different cell membrane receptors (52), our results suggest that they may also bind to the same receptor, indicating that their signaling may be somehow related. It is likely, then, that the inducement of skeletal muscle fibrosis by TGF-β1 is partially mediated by its interaction with MSTN. However, the mechanism by which TGF-β1 interacts with MSTN to cause fibrosis warrants further investigation.

Satellite cells serve as a reservoir of myogenic progenitor cells for the repair and maintenance of skeletal muscle. MSTN negatively regulates self-renewal and differentiation of satellite

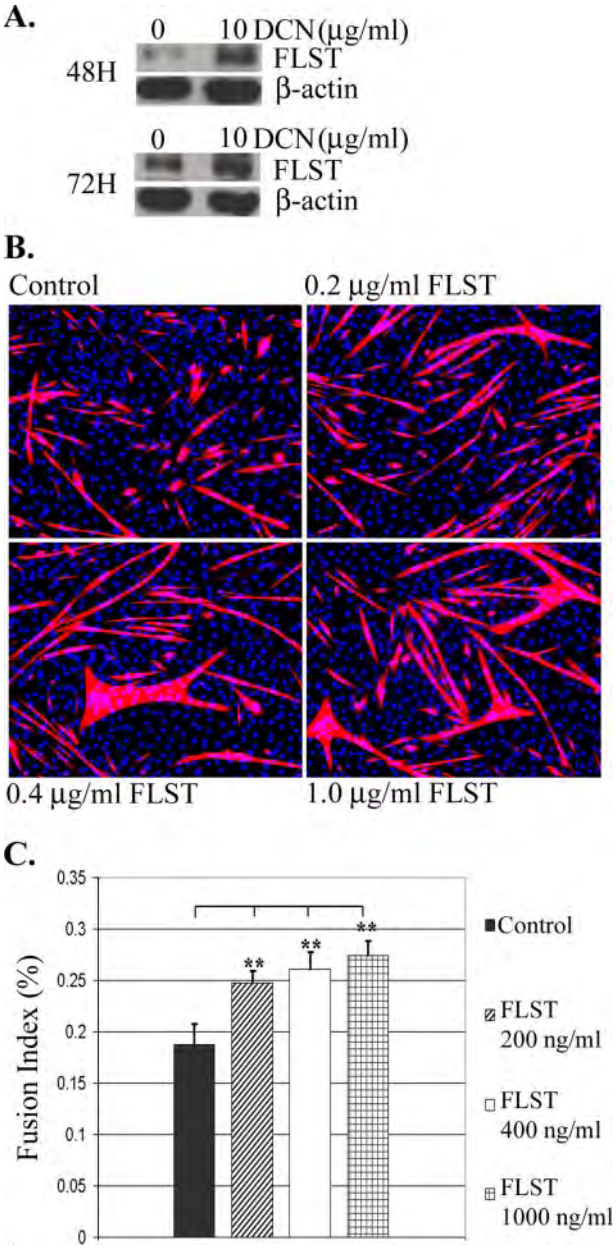

FIGURE 7. **The elevated FLST expression by DCN and the capacity of FLST to enhance myogenic differentiation of C2C12 myoblasts.** *A*, DCN increased the expression of FLST in C2C12 myoblasts 48 and 72 h after treatment. *B*, immunofluorescence analysis of myotubes. C2C12 myoblasts were maintained in DM for 5 days in the presence of different concentrations of FLST. Myotubes were double-labeled with an antibody recognizing skeletal myosin heavy chain and with the fluorescent nuclear dye Hoechst 33258 (magnification, ×100). *C*, fusion indexes were calculated to evaluate the degree of C2C12 myoblast differentiation upon FLST stimulation.

cells (54) and decreases the expression of members of the basic helix-loop-helix muscle regulatory factors (MRF) (MyoD, Myf5, mrf4, and myogenin) (43, 55). MSTN$^{-/-}$ mice show an increased number of satellite cells activated and differentiated toward a myogenic lineage (54). In this study, our data demonstrate that MSTN$^{-/-}$ mice contain regenerating myofibers with significantly larger diameters than WT mice at 2 and 4 weeks after GM laceration. The increased number of satellite cells in MSTN$^{-/-}$ mice could, in part, explain the enhanced regeneration revealed by the larger diameter of regenerated myofibers in MSTN$^{-/-}$ mice compared with WT mice. Indeed, it has been reported that blocking MSTN signals by isolating myoblasts from transgenic mice carrying the mutated MSTN receptor results in improved success of myoblast transplantation in *mdx* mice compared with normal myoblasts (56). Our results show that MSTN$^{-/-}$ LTP more readily undergo myogenic differentiation *in vitro* and regenerate skeletal muscle *in vivo* in a more effective manner than wild-type cells.

Furthermore, high levels of MSTN protein have been reported within necrotic fibers in the skeletal muscles of rats damaged by notexin (57), and Western blot analysis revealed the up-regulation of MSTN protein at early time points following notexin-induced injury in rat skeletal muscle (58). Interestingly, it has been shown that MSTN interferes with the chemotaxis of macrophages *in vitro* (51); recombinant MSTN protein significantly reduces the migration of macrophages and myoblasts toward chemoattractants *in vitro*, which likely promotes skeletal muscle regeneration (51). These results suggest that MSTN could impede recruitment of macrophages and myoblasts into the injured site *in vivo*. Macrophages infiltrate damaged tissue to remove debris that could hinder muscle regeneration. Macrophages also secrete a variety of growth factors and cytokines that have chemotactic and/or mitogenic effects on muscle precursor cells, thereby accelerating muscle regeneration (59–63). Compared with WT mice, MSTN$^{-/-}$ mice have shown elevated recruitment of macrophages and myoblasts and an accelerated inflammatory response after muscle injury (51). These results suggest that the earlier initiation of skeletal muscle regeneration in the injured skeletal muscle of MSTN$^{-/-}$ mice compared with the injured muscle of WT mice may be due, in part, to accelerated removal of muscle debris. When we monitored the expression of MSTN at the injured site for up to 30 days after injury, we observed an intense expression of MSTN in the cytoplasm of degenerative myofibers 1 and 3 days after laceration. On day 5 after injury, MSTN signal was detected in the cytoplasm of regenerating myofibers. Our results show that the MSTN signal decreases with maturation of regenerating myofibers. Interestingly, there is strong MSTN immunostaining in regenerating small myotubes lacking basal lamina 7 and 14 days post-injury. During skeletal muscle healing (following active muscle regeneration at early time points after injury) fibrosis initiates ~1 week post-injury, and peaks at 4 weeks (10, 15, 64). Li *et al.* (20) reported that some regenerating myofibers probably differentiate into myofibroblasts to contribute the formation of fibrosis. This correlation between fibrosis development and increased MSTN and TGF-β1 expression (20) in the early phase of healing may suggest the differentiation of regenerating myotubes/myofibers into myofibroblasts and a potential interaction between TGF-β1, MSTN, and DCN, as previously hypothesized (65).

MSTN-positive signals were also seen within the nuclei of the newly formed fibers at 5, 7, and 10 days post-injury. The nuclear localization of MSTN is supported by previous studies indicating that MSTN was detected in the nuclei of myoblasts and myotubes (66). Consequently, MSTN protein might modulate the muscle fiber regeneration process through the early events of phagocytosis and inflammation (57) and later control myofiber maturation. In this way, MSTN seems to act as a reg-

ulatory molecule that is produced by the tissue to specifically suppress and control the size of muscle growth and development (67).

DCN, a small chondroitin-dermatan sulfate leucine-rich proteoglycan, exists ubiquitously in the ECM. Due to its binding to and inhibition of TGF-β1, DCN has been used as a potent anti-fibrosis agent in various organs and tissues (26, 27, 37–40), including skeletal muscle (26, 27). However, the ability of DCN to regulate MSTN activity is still unknown. DCN, which is composed of a core protein and a single glycosaminoglycan chain (68, 69), has the ability to bind to TGF-β1 due to the fact that the core protein of DCN contains two binding sites for TGF-β1 (70). Similarly, Miura *et al.* (65) have shown that DCN, or the core protein of DCN, directly binds to active MSTN molecules to block MSTN-mediated inhibition of C2C12 myoblast proliferation. The actual location of the MSTN binding site in the DCN core protein and evidence that shows whether TGF-β1 and MSTN competitively bind to DCN are topics for further investigation. Of further interest is the possibility that DCN may regulate MSTN by influencing another intermediate molecule like FLST, an antagonist of MSTN (46). Our results not only show that DCN reduces the effects of MSTN on fibroblasts and myoblasts, but also indicates that it stimulates the expression of FLST in C2C12 myoblasts. Exogenous FLST then stimulates C2C12 myoblast differentiation, which is probably due to FLST's neutralization of endogenous MSTN. These results indicate that the effect of DCN on MSTN may be related to the up-regulation of FLST, which would consequently suppress MSTN activity. Nevertheless, more experiments that would, for example, examine the effect of DCN on FLST knock-out cells, need to be done to establish the role of FLST in DCN-inhibited MSTN activity. Furthermore, we have shown that TGF-β1 probably plays a role in the MSTN signaling pathway, because TGF-β1-soluble receptor antagonizes, at least in part, the effect of myostatin on muscle cells. Overall, DCN probably regulates MSTN activity via three ways: (i) directly binding MSTN, (ii) indirectly down-regulate MSTN by binding to TGF-β1, and (iii) indirectly down-regulating MSTN by stimulating FLST expression.

In summary, our results suggest the following: (i) MSTN stimulates the formation of fibrosis in skeletal muscle after injury, (ii) TGF-β1 and MSTN up-regulate the expression level of each other, and (iii) DCN is capable of inhibiting MSTN activity as it does for TGF-β1. These results, combined with the fact that TGF-β1 plays a key role in skeletal muscle fibrosis and that DCN reduces fibrosis in injured skeletal muscle, suggest that TGF-β1 and MSTN probably act together; they synergistically amplify the fibrotic process in injured or diseased skeletal muscles resulting in greater fibrosis than either could induce individually.

Our findings may help to further increase the understanding of the mechanism by which $MSTN^{-/-}$ mice show decreased fibrosis and enhanced regeneration after injury and suggest that the inhibition of MSTN might be a new therapeutic approach for improving skeletal muscle healing through enhancement of regeneration and reduction of fibrosis.

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DECORIN INTERACTS WITH MYOSTATIN ACTIVITY – IMPLICATIONS FOR SKELETAL MUSCLE HEALING

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Introduction:

Skeletal muscle injuries are one of the most common injuries occuring in field of sports medicine. Muscle injuries can heal spontaneously via regeneration, but fibrosis usually impedes this process which results in incomplete functional recovery. We have demonstrated that TGF-β1 plays a major role in the initiation of fibrosis in injured muscle [1]. Decorin, a small chondroitin-dermatan sulphate leucine-rich proteoglycan, has been shown to improve skeletal muscle healing histologically and physiologically through a concomitant increase in muscle regeneration and a decrease in fibrosis [2]. It is widely accepted that the antifibrotic effect of decorin primarily results from its capability of directly binding to and neutralizing TGF-β1 [3]. The identification of a new TGF-β family member, myostatin (MSTN) [4], has inspired us to further explore the mechanism by which decorin improves skeletal muscle healing. Our unpublished data showed that 1) MSTN stimulates proliferation and myofibrotic differentiation of fibroblasts in vitro; 2) that the injured skeletal muscle of MSTN knockout (MSTN-/-) mice contains significantly less fibrous scar tissue than observed in the injured muscle of normal wild-type (WT) mice and 3) regenerating myofibers of MSTN-/- mice are significantly larger in diameter than those in the injured muscle of WT mice. Thus MSTN, like TGF-β1, appears to play an important role in fibrosis. We performed this study to investigate whether decorin could inhibit the activity of MSTN as it does with TGF-β1.

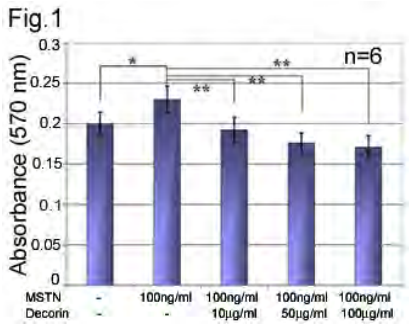
Methods:

Proliferation assay and Western blot analysis: PP1 fibroblasts were plated onto collagen-coated 96-well plates for cell proliferation analysis. Following overnight attachment, normal medium was replaced with serum free medium plus serum replacement (Sigma, St Louis, MO) in the presence of varying concentrations of decorin and 100ng/ml of recombinant human MSTN. After an additional incubation for 48 h, an MTT cell proliferation assay kit (Roche Diagnostics, Germany) was used to measure cell proliferation (n = 6) following instructions from the manufacturer. Western blot was used to examine β-actin and MSTN expression. To test whether decorin neutralized the inhibitory effect of MSTN on myogenic cell differentiation, C2C12 myoblasts were seeded in 12-well plates at a density of 10,000cells/well. After overnight incubation, medium was replaced with fresh differentiation medium (DM) containing DMEM, 2% HS, and 1% P/S, with or without addition of 1μg/ml MSTN. This was concomitant with the addition of 0-100 μg/ml decorin (n = 3). Cells were cultured for another 5 days. DM, MSTN, and decorin were changed every other day.

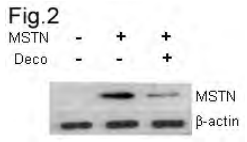
Injury model: All experiments in this study were in accordance with research protocols approved by the ARCC of Children's Hospital of Pittsburgh. MSTN-deficient mice (MSTN-/-) and C57BL/6J wild-type mice (control) were used to establish an injury model of skeletal muscle laceration [2]. GMs of both MSTN-/- and wild-type mice were harvested 2 weeks after laceration. Decorin immunostaining was performed to evaluate decorin expression in injured skeletal muscle. Student's *t*-test or one way ANOVA were used to determine significance ($P < 0.05$).

Results:

Blockade of MSTN effect on fibroblasts by Decorin:. After PP1 fibroblasts were incubated with MSTN and varying concentrations of decorin for 48 h, MTT assay revealed that 0.1μg/ml MSTN significantly stimulated PP1 fibroblast proliferation whereas the addition of decorin significantly repressed the MSTN stimulatory effect on PP1 proliferation (Fig 1).

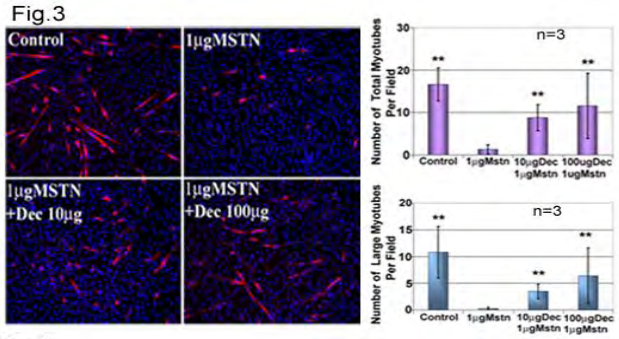


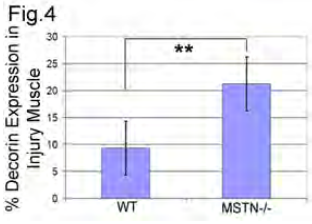
Decorin blocks MSTN autocrine expression in fibroblasts: After 48 h incubation, PP1 fibroblasts did not express detectable MSTN protein, and PP1 fibroblasts treated with MSTN showed a high level of MSTN expression. However, MSTN expression in PP1 fibroblasts treated with both MSTN and decorin was reduced to only a detectable level (Fig 2).



Blockade of MSTN effect on C2C12 myoblast differentiation by Decorin
Following a 5-day incubation, controls showed widespread skeletal muscle heavy chain (MHC) positive myotubes whereas cells treated with MSTN alone contained few myotubes (Fig 3). The addition of decorin reversed the inhibition of MSTN on myogenic differentiation (Fig 3). Quantification showed that decorin treatment significantly promoted C2C12 myoblast fusion and increased the number and size of the myotubes in the presence of MSTN (Fig 3) in a dose dependent manner

Elevated decorin expression in injured MSTN-/- mice
Immnunohistochemical staining revealed that there was a significantly higher level of decorin expression in the regenerating skeletal muscle of MSTN-/- mice than that of WT mice 2 weeks after injury (Fig 4).





Discussion:

In this study, we showed that decorin blocked the effects of MSTN on both fibroblasts and myoblasts in vitro. Decorin reduced the stimulating effects of MSTN on fibroblasts and the MSTN autocrine expression of fibroblasts. Moreover, decorin rescued the inhibitory effect of MSTN on myoblast differentiation. The ability of decorin to bind to TGF-β1 is due to the fact that core protein of decorin contains two binding sites for TGF-β1. Similarly, it has been recently shown that decorin, or the core protein of decorin, can directly bond to active MSTN molecules to block MSTN-mediated inhibition of C2C12 myoblast proliferation [5]. The location of the MSTN binding site in the decorin core protein and whether TGF-β1 and MSTN competitively bind to decorin are topics for further investigation. These findings provide us a better understanding of how decorin can improve skeletal muscle healing after injury.

Acknowledgments: This work was supported by funding from the Henry J. Mankin Endowed Chair for Orthopaedic Research at the University of Pittsburgh, the William F. and Jean W. Donaldson Chair at Children's Hospital of Pittsburgh, the Hirtzel Foundation, and the National Institutes of Health (R01 AR47973.). We thank Dr. Se-Jin Lee (Johns Hopkins University) for providing us the MSTN-/- mice.

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**Department of Bioengineering, University of Pittsburgh

FOLLISTATIN IMPROVES SKELETAL MUSCLE HEALING AFTER INJURY

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INTRODUCTION

Skeletal muscle injuries account for 10 to 30% of all sports related injuries. Muscle injuries can heal via spontaneous regeneration; however, as a result of severe injury, incomplete functional recovery can increase the time an athlete is off the field. Decorin has been shown to effectively improve skeletal muscle healing by reducing fibrosis and accelerating skeletal muscle regeneration [1]. Aside from decorin, Follistatin (FLST) has been drawing more attention do to the fact that increasing knowledge about this molecule reveals it as a promising new target for therapeutic improvement of skeletal muscle healing. FLST was initially described as an inhibitor of follicle-stimulating hormone decades ago. More and more evidence revealed that FLST is capable of binding and neutralizing many members of the TGF-β superfamily such as myostatin (MSTN) and activin [2, 3]. Recently, it was found that FLST overexpression mice (FLST OE) showed dramatic increase in skeletal muscle mass compared to wild-type control mice [4]. Activin is implicated in the formation of fibrosis in many tissues and organs such as skin, liver, and kidney [2, 3]. Furthermore, our unpublished data demonstrated that MSTN is a fibrosis stimulator in the skeletal muscle and blocking MSTN significantly reduces the formation of fibrosis in injured skeletal muscle of mice. Taken together, we hypothesize that FLST may improve the healing of injured skeletal muscle. In this study, transgenic FLST OE mice were used to investigate the influence of FLST on skeletal muscle healing.

METHODS

Myoblast differentiation assay: C2C12 myoblasts were plated onto collagen-coated 12-well plates with normal medium overnight. The following day, the medium was replaced with low serum medium (2% horse serum) plus different concentrations of FLST (Sigma, St. Louis, MO). The cells were cultured for 6 additional days. Medium and recombinant FLST protein were changed every other day. Myotubes were monitored by myosin heavy chain (MHC) immunostaining, and the fusion capacity of C2C12 myoblasts was evaluated by determining the number of myonuclei per myotube (n = 3).

Western blot: C2C12 myoblasts were plated onto collagen-coated 6-well plates. Following overnight attachment, normal medium was replaced with low serum medium in the presence of decorin (Sigma, St. Louis, MO). Protein samples were collected at 48 and 72h and separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Goat anti-follistatin antibody (Santa Cruz Biotechnology, INC. Santa Cruz, CA) was used to detect FLST expression in C2C12 myoblasts

Animal model: All experiments in this study were approved by the Children's Hospital of Pittsburgh IACUC. C57BL/6J wide-type (WT) and FLST OE mice (7 to 8 weeks of age) were used for all experiments (n = 6). Both gastrocnemius muscles (GMs) of each mouse underwent bilateral laceration [1]. The GMs were harvested 4 weeks after laceration, and Masson's Trichrome staining (nuclei [black], muscle [red], collagen [blue]) was performed to identify fibrous scar tissue in the injured muscles. Northern Eclipse software (Empix Imaging, Inc.) was used to measure areas of fibrous scar tissue and regenerated muscle within the injury site.

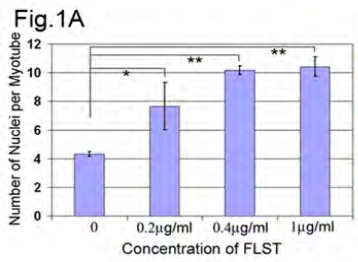
Statistics: One-way analysis of variance (ANOVA) followed by post hoc Tukey's multiple comparison test or Student's *t* test was used to determine significance ($P < 0.05$) throughout this study. (*) represents that $P < 0.05$, and (**) represents that $P < 0.01$;

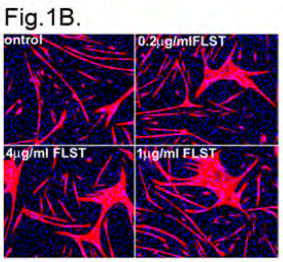
RESULTS

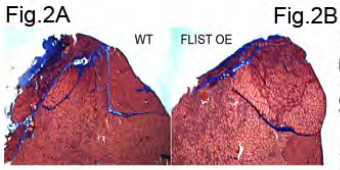
FLST induces large myotube formation in vitro: Our results reveal the ability of FLST to stimulate myogenic differentiation as shown in Fig 1. Myotubes treated with FLST were larger and contained more nuclei than the control (Fig. 1A). FLST treatment leads to a significant increase in the number of nuclei per myotubes in a dose dependent manner (Fig. 1B) compared to control, suggesting that FLST promotes myogenic fusion and accelerates the maturation of myotubes.

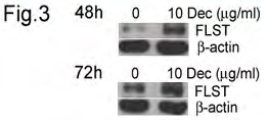
Reduced fibrosis in FLST OE mice after GM laceration: The results of Masson's Trichrome histochemistry revealed significantly less fibrous scar tissue in the FLST OE GMs than in the WT GMs (Fig. 2A, B).

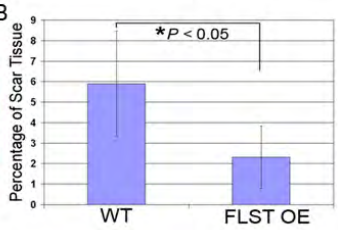
Decorin stimulates FLST expression in C2C12 myoblasts: We found that decorin up-regulated FLST expression in C2C12 myoblasts after 48h and 72h of culture (Fig.3).











DISCUSSION

We have demonstrated that TGF-β1 plays a significant role in both the initiation of fibrosis and the induction of myofibroblastic differentiation by myogenic cells in injured skeletal muscle [6]. Although, FLST improves skeletal muscle healing, it does not seem to block fibrosis in injured skeletal muscle through inhibition of TGF-β1. Our result showed that FLST failed to block the inhibition that TGF- β1 has on myogenic differentiation (data not shown). Most likely, FLST inhibits the formation of fibrosis by antagonizing the activities of activin and MSTN [2, 3]. FLST overexpression improves skeletal muscle healing by reducing fibrosis. Although FLST significantly increases myoblast differentiation in vitro, FLST OE mice did not show increased diameter of regenerating myofibers compared to WT mice at 4 weeks after GM laceration. This suggests that FLST is involved with an alternative mechanism for skeletal muscle healing. Moreover, we also showed that decorin treatment elevated FLST expression in C2C12 myoblasts. It seems likely that, apart from neutralizing TGF- β1, decorin also reduces fibrosis in injured skeletal muscle by up-regulating the intermediate molecule, FLST. The skeletal muscle healing process is a complex process, in which many molecules interact with each other, and our findings provide insight into one aspect of this network.

ACKNOWLEDGMENTS

This work was supported by funding from the Henry J. Mankin Endowed Chair for Orthopaedic Research at the University of Pittsburgh, the William F. and Jean W. Donaldson Chair at Children's Hospital of Pittsburgh, the Hirtzel Foundation, and the National Institutes of Health (R01 AR47973). The FLST OE mice were a gift from Dr. Se-Jin Lee (Johns Hopkins University).

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Suramin can enhance the skeletal muscle healing by blocking myostation

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INTRODUCTION:

Muscle injuries are very common musculoskeletal problems encountered in sports medicine. Although this type of injury is capable of healing, complete functional recovery is hindered by the development of scar tissue formation triggered by TGF-β1 [1]. We already reported suramin can effectively prevent the formation of fibrotic scar and block the proliferative effect of TGF-β1 on fibroblasts and can stimulate the differentiation on myogenic cells *in vitro* (unpublished data). Thus suramin can enhance muscle regeneration in the lacerated and strain-injured muscle [2, 3]. Furthermore we also reported blockade of myostatin (MSTN), a member of TGF-β super family, by decorin, other anti-fibrotic agent, showed enhancement of the fusion on myoblasts and inhibitory effect of fibroblast proliferation *in vitro* [4]. This finding brought us more interest to investigate another pathway of suramin to regulate fibroblasts and myoblasts by blocking the effect of MSTN. We performed this study to examine whether suramin would block MSTN's proliferative effect on fibroblasts and inhibitory effect of myoblasts differentiation *in vitro* and reduce the expression of MSTN in injured muscle to improve muscle healing *in vivo*, using an animal model of muscle contusion.

MATERIALS AND METHODS:

3T3 cell proliferation assay: 3T3 fibroblasts were cultured in 96 well plates (n=5) with DMEM containing 2% serum replacement (Sigma, St Louis, MO) and different concentration of MSTN (0 and 1 μg/ml) and suramin (0 and 50 μg/ml). Three days after incubation CellTiter Cell Proliferation Assay kit (Promega, Madison, WI) was used to measure cell proliferation.

C2C12 cell differentiation assay: C2C12 myoblasts were cultured in 24 well plates (n=4) with differentiation medium (DMEM containing 2% horse serum and 1% penicillin/streptomycin) containing different concentration of MSTN (0 and 1 μg/ml) and suramin (0, 1,and 25 μg/ml). Three days after incubation immunocytochemistry of myosin heavy chain was done and the fusion index was assessed by counting the number of nuclei in differentiated myotubes as a percentage of the total number of nuclei.

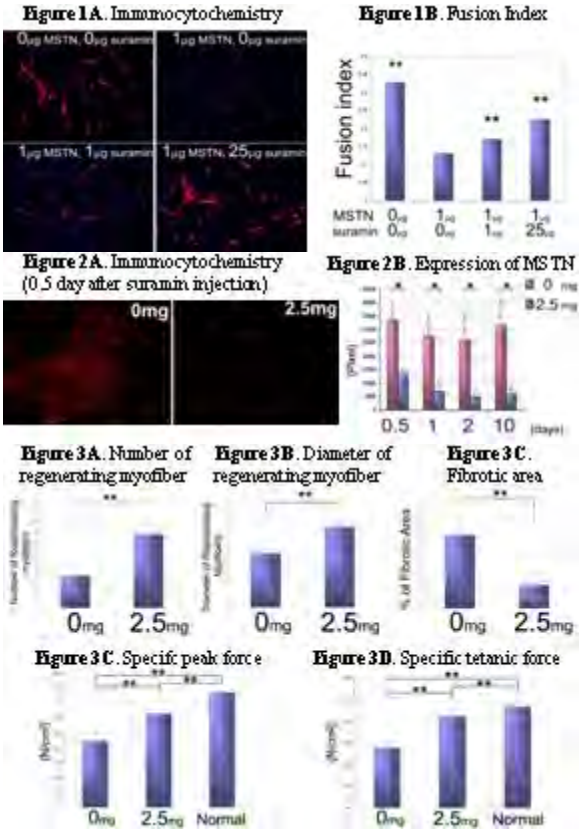
Animal model: The muscle contusion model was developed in tibialis anterior muscle of normal wild-type mice. Different concentrations of suramin (0 and 2.5 mg in 20μl of Phosphate-buffered solution [PBS]) were injected intramuscularly two weeks after injury (five mice in each group). Cryostat sections of muscles were obtained and histologically stained (hematoxlin and eosin stain (H&E) and Masson's Trichrome stain) to evaluate the regeneration by counting the number of centronucleated regenerating myofibers and measuring fibrosis four weeks after injury. Specific peak force and specific tetanic force were mesured as physiological tests to evaluate functional recovery after muscle injury using same protocol as above (five mice in each group). Furthermore immunohistochemistry of MSTN was performed at different time point (0.5, 1, 2, 10 days after suramin (0 and 2.5 mg) injection) to evaluate the expression of MSTN (five mice in each group at each time point). Northern Eclipse software (Empix Image, Inc.) was used to quantify the total fibrotic area and expression of MSTN. Statistical analysis was performed with student's t-test or ANOVA. Statistical significance was defined as $P < .05$ (*: P<.05, **: P<.01).

RESULTS:

Suramin blocked the proliferative effect of MSTN on fibroblasts and the inhibitory effect of MSDN of myoblast differentiation: MSTN treatment significantly promoted the proliferation on fibroblasts and the differentiation on myoblasts. However, suramin treatment significantly blocked both of those MSTN's effects and moreover suramin treatment stimulated the fusion on myoblasts in a dose-dependent manner in the presence of MSTN (Fig. 1A, B).

Suramin inhibits the myostatin expression in injured skeletal muscle: Suramin (2.5 mg) injection 2 weeks after contusion injury effectively inhibited the expression of MSTN when compared with the control group (0 mg) at different time points (0.5, 1, 2, 10 days after suramin injection) (Fig. 2A, B).

Suramin enhances muscle regeneration and decreases fibrosis and improves functional recovery after contusion injury: We observed a significant increase in the number and in the diameter of regenerating myofibers in the suramin treated group (2.5 mg) when compared with the control group (0mg) (Fig. 3A, B,). Moreover suramin treated group showed significantly less fibrotic area than control group (Fig. 3C). Furthermore the suramin treatment showed significant advantage in physiological evaluation (specific peak force and specific tetanic force) compared to control group.



DISCUSSION:

We have reported that suramin can effectively prevent muscle fibrosis and enhance muscle regeneration by blocking TGF-β1 after laceration and strain injury [2, 3]. Nevertheless, whether suramin would regulate the effect of MSTN, negative regulator of muscle growth, to improve muscle healing was still unknown. This is the first study to show that suramin down-regulates the proliferation on fibroblasts and up-regulates the differentiation on myoblasts by neutralizing MSTN. Moreover suramin injection in injured skeletal muscle effectively inhibited the expression of MSTN and enhanced the muscle regeneration and reduced the fibrosis *in vivo*. These results may reveal the hidden mechanism by which suramin improve the muscle healing after injuries. We have reported important anti-fibrotic molecules such as Decorin to improve injured skeletal muscles. However suramin has been already approved by FDA and this makes suramin more suitable agent for the therapy of muscle injury. Our findings may contribute to the development of biological therapies for muscle injury.

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Improved Muscle Healing After Contusion Injury by the Inhibitory Effect of Suramin on Myostatin, a Negative Regulator of Muscle Growth

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Improved Muscle Healing After Contusion Injury by the Inhibitory Effect of Suramin on Myostatin, a Negative Regulator of Muscle Growth

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Background: Muscle contusions are the most common muscle injuries in sports medicine. Although these injuries are capable of healing, incomplete functional recovery often occurs.

Hypothesis: Suramin enhances muscle healing by both stimulating muscle regeneration and preventing fibrosis in contused skeletal muscle.

Study Design: Controlled laboratory study.

Methods: In vitro: Myoblasts (C2C12 cells) and muscle-derived stem cells (MDSCs) were cultured with suramin, and the potential of suramin to induce their differentiation was evaluated. Furthermore, MDSCs were cocultured with suramin and myostatin (MSTN) to monitor the capability of suramin to neutralize the effect of MSTN. In vivo: Varying concentrations of suramin were injected in the tibialis anterior muscle of mice 2 weeks after muscle contusion injury. Muscle regeneration and scar tissue formation were evaluated by histologic analysis and functional recovery was measured by physiologic testing

Results: In vitro: Suramin stimulated the differentiation of myoblasts and MDSCs in a dose-dependent manner. Moreover, suramin neutralized the inhibitory effect of MSTN on MDSC differentiation. In vivo: Suramin treatment significantly promoted muscle regeneration, decreased fibrosis formation, reduced myostatin expression in injured muscle, and increased muscle strength after contusion injury.

Conclusion: Intramuscular injection of suramin after a contusion injury improved overall skeletal muscle healing. Suramin enhanced myoblast and MDSC differentiation and neutralized MSTN's negative effect on myogenic differentiation in vitro, which suggests a possible mechanism for the beneficial effects that this pharmacologic agent exhibits in vivo.

Clinical Relevance: These findings could contribute to the development of biological treatments to aid in muscle healing after experiencing a muscle injury.

Keywords: muscle contusion injury; suramin; myostatin; muscle regeneration; fibrosis

Muscle injuries are common musculoskeletal problems encountered in sports medicine clinics. Muscle contusion, produced by the impact of a nonpenetrating object,[8] is one of the most common muscle injuries.[3] Current therapies including RICE (rest, ice, compression, and elevation), immobilization, as well as active and passive range of motion exercise are the norm for treatment; however, complications such as muscle atrophy, contracture formation, and pain leading to functional and structural deficits often occur after severe muscle injury.[8,24,25,38] Optimal treatment strategies have not yet been clearly defined.

We have observed that the healing process of injured skeletal muscle in animal models consists of 3 distinct phases:

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degeneration and inflammation, regeneration, and fibrosis.[13,15,16,26] The first phase, which occurs in the first few days after injury, is characterized by local swelling at the injury site, the formation of a hematoma, necrosis of muscle tissue,[20,23] degeneration, and an inflammatory response, which consists of the infiltration of activated macrophages and T-lymphocytes into the injured tissue. The next phase, regeneration, usually occurs 5 to 10 days after injury and includes phagocytosis of the damaged tissue and regeneration of the injured muscle. This phase is promoted by the release of several growth factors that regulate myoblast proliferation and differentiation, produce connective tissue and scar tissue, and induce capillary ingrowth at the injury site.[1,4,17]

The final phase, the formation of scar tissue (fibrosis), usually begins between the second and third week after injury. The formation of scar appears to be the end product of the muscle repair process and hinders full muscle regeneration. We have previously reported that transforming growth factor–beta 1 (TGF-β1) is a major factor in triggering the fibrotic cascade within injured skeletal muscle.[30,31] With this in mind, we have focused on the use of antifibrotic agents (such as decorin and γ-interferon) that inhibit TGF-β1 expression, reduce scar tissue formation, and consequently improve muscle healing after injury.[12,13] Administration of decorin after muscle injury showed improvement of muscle healing both histologically and physiologically.[13] The injection of γ-interferon into injured skeletal muscle also showed similar effects.[12] Despite these compelling findings, which were derived from various murine injury models, the fact that decorin is not clinically available limits its translation to human subjects and γ-interferon demonstrates serious side effects in spite of US Food and Drug Administration (FDA) approval. A recently published study has shown that myostatin (MSTN), a member of the TGF-β superfamily and a negative regulator of muscle growth, stimulates scar tissue formation after skeletal muscle injury in vivo.[48] It was also shown in this study that decorin could inhibit MSTN's activity in vitro.[48] Because both decorin and suramin have the ability to inhibit TGF-β1 activity,[5,6,13] we hypothesize that suramin may also possess a similar anti-MSTN activity as decorin.

Suramin, an antiparasitic and antineoplastic agent, can inhibit TGF-β1's ability to bind to its receptors and has been shown to enhance muscle regeneration after strain and laceration injuries.[5,6] We pursued the use of suramin due to the fact that it is currently FDA-approved and could thereby be used clinically more readily than decorin. In the current study, we examine whether suramin can promote differentiation of myoblasts and muscle-derived stem cells (MDSCs) and neutralize the effect of MSTN in vitro. We also investigate whether suramin can improve muscle healing after muscle contusion, a common muscle injury, by enhancing regeneration and reducing fibrosis in vivo.

MATERIALS AND METHODS

In Vitro Potential of Suramin to Induce Myogenic Differentiation

Effect of Suramin on C2C12 Myoblasts. C2C12 cells, a well-known myoblast cell line, were cultured with Dulbecco's modified Eagle's medium (DMEM) (Invitrogen, Carlsbad, California) containing 10% fetal bovine serum (FBS) (Invitrogen), and 1% penicillin/streptomycin (P/S) (Invitrogen). Cells were plated at a density of 10 000 cells/well into 12-well plates. After a 24-hour incubation period, the medium was completely removed and low serum-containing medium (DMEM, 2% horse serum [HS] [Invitrogen], and 1% P/S) was added with varying concentrations of suramin (0, 0.25, 2.5, 25 μg/mL) (Sigma, St Louis, Missouri). Medium was replaced with fresh medium (containing the same concentrations of suramin) every 2 days. All the cells were grown at 37°C in 5% CO_2 for a total of 4 days.

Effect of Suramin on MDSCs Isolated From Skeletal Muscle. MDSCs were isolated from 3-week-old male mice (C57BL10J+/+) via the previously described modified preplate technique.[31,35,36] Muscle-derived stem cells were cultured in proliferation medium (PM) containing DMEM, 10% FBS, 10% HS, 1% P/S, and 0.5% chick embryo extract (Sera Laboratories International, West Sussex, United Kingdom). Cells were plated at a density of 10 000 cells/well into 12-well plates. After a 24-hour incubation period, the medium was completely removed and low-serum medium was added with different concentrations of suramin (0, 1, 10, 100 μg/mL) for the next 24 hours. Medium was replaced with fresh low-serum medium for 1 more day. All the cells were grown at 37°C in 5% CO_2 for a total of 3 days.

Ability of Suramin to Neutralize MSTN's Inhibition of MDSC Differentiation. Muscle-derived stem cells were cultured in PM and plated at a density of 5000 cells/well into 24-well plates. After a 24-hour incubation period, the medium was removed and low serum-containing medium was added with varying concentrations of suramin (0, 1, 10, or 100 μg/mL) and MSTN (0 or 100 ng/mL) for the next 24 hours. Medium was replaced with fresh low serum medium containing the same concentrations of MSTN for 1 additional day. All the cells were grown at 37°C in 5% CO_2 for a total of 3 days.

Immunocytochemistry

To quantify the differentiation of the C2C12 cells and MDSCs, cells were fixed in cold methanol for 2 minutes and washed in Dulbecco's phosphate-buffered saline (PBS) for 10 minutes at room temperature (RT). Samples were washed 3 times in PBS, then incubated in blocking buffer (10% HS) for 30 minutes at RT. Cells were incubated overnight at 4°C with primary antibodies (monoclonal antiskeletal myosin [fast] clone MY-32 [Sigma]) in 2% HS. After washing in PBS, samples were incubated with the secondary antibody (goat antimouse IgG conjugated with Cy3 [Sigma]) in 2% HS for 1 hour at RT. The cell nuclei were stained with a Hoechst 33258 dye for 10 minutes at RT. Fusion index (ratio of nuclei in myotubes to all nuclei) was calculated to evaluate the myogenic differentiation capacity of cells.

Evaluation of the Histologic and Physiologic Effects of Suramin on Muscle Healing After Contusion Injury In Vivo

Animal Model. The policies and procedures followed for the animal experimentation performed in these studies are

in accordance with those detailed by the US Department of Health and Human Services and the National Institutes of Health *Guide for the Care and Use of Laboratory Animals.* Our Institutional Animal Care and Use Committee approved the research protocol used for these experiments (protocol No. 19/05). An animal model of muscle contusion was developed in normal mice (C57BL6J+/+, Jackson Laboratory, Bar Harbor, Maine) based on previously described studies.[8,26] Thirty-three mice, aged 8 to 10 weeks with weights of 21.0 to 26.3 g, were used in this experiment.

The mice were anesthetized with 1.0% to 1.5% isoflurane (Abbott Laboratories, North Chicago, Illinois) in 100% O_2 gas. The mouse's hindlimb was positioned by extending the knee and plantar flexing the ankle 90°. A 16.2-g, 1.6-cm stainless steel ball (Small Parts Inc, Miami Lakes, Florida) was dropped from a height of 100 cm onto the impactor that hit the mouse's tibialis anterior (TA) muscle. The muscle contusion made by this method was a high-energy blunt injury that created a large hematoma and was followed by massive muscle regeneration,[8,26] healing processes that are very similar to those seen in humans.[10] The mice were divided into 4 groups (6 mice/group) on the basis of the different concentrations of suramin to be injected (0, 2.5, 5, and 10 mg in 20 μL of PBS) after creating the contusion injury. Suramin was injected 2 weeks after injury. All animals were sacrificed to evaluate the healing histologically and physiologically at 4 weeks after injury. Three mice (6 muscles) per group were assessed histologically, 3 mice (6 muscles) per group were assessed physiologically, and 3 mice were used as normal controls in the physiologic tests.

Furthermore, 2 concentrations of suramin (0 and 2.5 mg in 20 μL of PBS) were injected 2 weeks after injury (3 mice/group). The mice of both groups were sacrificed 2 days after injection for histologic analysis of MSTN expression.

Evaluation of Muscle Regeneration After Suramin Therapy. Tibialis anterior muscles that were used for histologic evaluation were isolated and frozen in 2-methylbutane precooled in liquid nitrogen and subsequently cryosectioned. Hematoxylin and eosin staining was done to monitor the number of regenerating myofibers within the injury sites treated with suramin (0, 2.5, 5, or 10 mg in 20 μL of PBS), and the results were compared among the different groups. Centronucleated myofibers were considered to be regenerating myofibers. Centrally nucleated myofibers are a sign of regeneration in injured and diseased muscle. Upon myoblast fusing into myotubes or with myofibers, the nuclei remain in the endomysial tube found in the center of the myofiber until it has reached maturity whereby the nuclei migrate to their final positions at the periphery of the mature fiber in the subsarcolemmal position. The central nucleation is easily identified by hematoxylin and eosin or trichrome staining.[11,37] Analysis of regenerating myofibers was performed using Northern Eclipse software (Empix Imaging Inc, Cheektawaga, New York). The total number of regenerating myofibers within the injury site was quantified by using 10 random fields selected from each sample in accordance with a previously described protocol.[12,13,26,33]

Evaluation of Fibrosis After Suramin Therapy. To measure areas of fibrotic tissue in the injury sites, Masson's trichrome staining (IMEB Inc, Chicago, Illinois) was performed. After Masson trichrome staining, the ratio of the fibrotic area to the total cross-sectional area was calculated to estimate the fibrosis formation by using Northern Eclipse software (Empix Imaging Inc). The ratio of the fibrotic area within the injury sites was quantified using a previously described protocol.[12,13,26, 3]

Evaluation of MSTN Expression After Suramin Therapy. To measure the MSTN expression in injured muscle, cryosectioned tissue was fixed in 4% formalin for 5 minutes followed by two 10-minute washes with PBS. The sections were then blocked with 10% HS for 1 hour at RT. Sections were incubated overnight at 4°C with primary antibodies (goat anti GDF-8 AF-788, R&D Systems Inc, Minneapolis, Minnesota) in 2% HS. After washing in PBS, samples were incubated with the secondary antibody (donkey anti-goat IgG conjugated with Alexa Fluor 555 [Invitrogen]) in 2% HS for 1 hour at RT.

The total MSTN positive area was calculated using Northern Eclipse software (Empix Imaging Inc) The total positive area within the injury site was quantified using a previously described protocol.[12,13,26,33]

Physiologic Evaluation of Muscle Contractile Properties After Suramin Therapy. Contusion injuries were made on TA muscles of both legs of normal mice and treated as described above. Four weeks after injury, muscles from both legs were tested to evaluate peak twitch and tetanic force. Tibialis anterior muscles were harvested bilaterally, placed in a vertical chamber that was constantly perfused with mammalian Ringer solution aerated with 95% O_2–5% CO_2 and maintained at 25°C. The distal attachment of the muscle was mounted to a glass tissue-support rod, and the proximal end of muscle on the tibia was connected to a force transducer and length servo system (Aurora Scientific, Aurora, Ontario, Canada). The muscles were stimulated by monophasic rectangular pulses of current (1 millisecond in duration) delivered through platinum electrodes placed 1 cm apart. The current was increased by 50% more than the current necessary to obtain peak force (250-300 mA) to ensure maximal stimulation. Using a micropositioner, muscles were first adjusted to their optimum length (Lo), defined as the length at which maximum isometric twitch tension was produced. Maximal tetanic force was assessed via a stimulation frequency of 75 Hz delivered in a 500-millisecond train. After the procedure, each muscle was weighed and specific peak twitch force and specific peak tetanic force were calculated and expressed in force per unit cross-sectional area (N/cm^2).

Statistical Analysis

All of the results from this study were expressed as the mean ± standard deviation. The result of MSTN expression in vivo was analyzed using the *t* test and all the other results were statistically analyzed using analysis of variance. Differences among the groups were analyzed by using Scheffé multiple comparisons (post hoc test). Statistical significance was defined as $P < .05$.

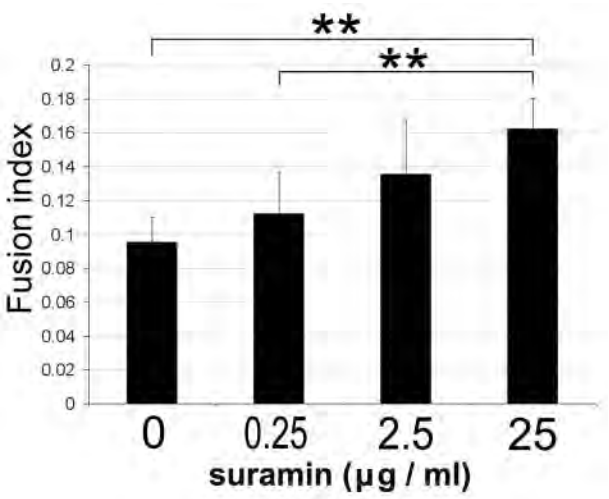

Figure 1. Fusion index was measured to evaluate C2C12 myoblast differentiation stimulated by different concentration of suramin (0, 0.25, 2.5, and 25 µg/mL). $^{**}P < .01$.

RESULTS

In Vitro Potential of Suramin to Induce Myogenic Differentiation

Effect of Suramin on Myoblasts. The effect of suramin on the differentiation of C2C12 cells is shown in Figure 1. The fusion index of C2C12 cells in the group treated with suramin (25 µg/mL) (0.162 ± 0.018) was significantly higher at 4 days of incubation as compared with the fusion index in the control group (0 µg/mL) (0.953 ± 0.015). Moreover, the high-dose suramin-treated group (25 µg/mL) displayed a significantly higher fusion index than the low-dose suramin-treated group (0.25 µg/mL). The effect of suramin to induce the differentiation of C2C12 appeared to be dose-dependent.

Effect of Suramin on MDSCs. Suramin also stimulated MDSC differentiation in a dose-dependent manner (Figure 2E). Suramin-treated MDSCs enhanced the differentiation of MDSCs after 3 days of incubation. Suramin-treated groups (10 and 100 µg/mL) (Figures 2C and 2D) showed significantly higher fusion indexes (0.228 ± 0.025, 0.347 ± 0.0456) than the control group (0 µg/mL) (Figure 2A) (0.149 ± 0.030). Among the suramin-treated groups, the high-dose suramin-treatment group (100 µg/mL) (Figure 2D) displayed a significantly higher fusion index when compared with the 2 lower-dose treatment groups (1 and 10 µg/mL) (Figures 2B and 2C).

Ability of Suramin to Neutralize MSTN's Inhibition of MDSC Differentiation. The group containing only MSTN (0 µg/mL suramin and 100 ng/mL MSTN) (Figure 3B) showed a significantly lower fusion index (0.091 ± 0.020) than the control group (0 µg/mL suramin and 0 ng/mL MSTN) (Figure 3A) (0.152 ± 0.035). All groups containing both suramin and MSTN (1, 10, and 100 µg/mL suramin and 100 ng/mL MSTN) (Figures 3C, 3D, and 3E) demonstrated significantly higher fusion indexes (0.153 ± 0.035, 0.162 ± 0.048, 0.279 ± 0.041) when compared with the group containing only MSTN (Figure 3B). Suramin appeared to neutralize MSTN's inhibitory effect on MDSC differentiation and, moreover, appeared to stimulate the fusion of MDSCs in a dose-dependent manner (Figure 3F).

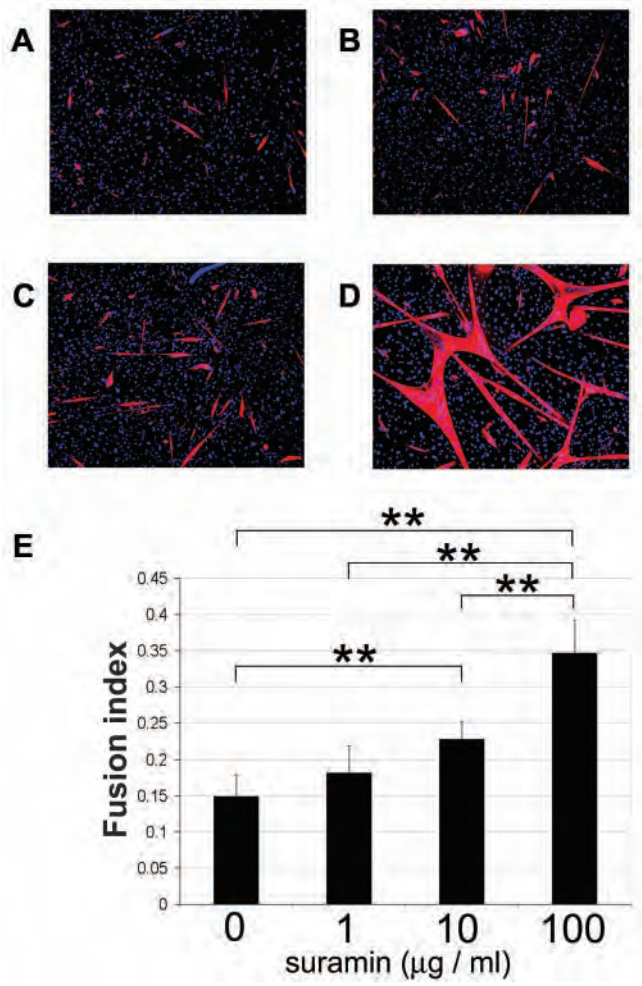

Figure 2. Immunocytochemical staining of muscle-derived stem cells (MDSCs) for fast myosin heavy chain at 3 days after incubation with different doses of suramin (A, 0 µg/mL; B, 1 µg/mL; C, 10 µg/mL; and D, 100 µg/mL). Myotubes are shown in red and nuclei are in blue (original magnification, ×200). E, comparison of fusion index of MDSC differentiation. $^{**}P < .01$.

Histologic and Physiologic Effects of Suramin on Muscle Healing After Contusion Injury In Vivo

Effect of Suramin Treatment on Muscle Regeneration After Contusion Injury. All the centronucleated regenerating myofibers present in the injured muscle were counted and compared among the groups (Figure 4E). The suramin-treated muscles displayed numerous regenerating myofibers at the contusion site. All the suramin-treated groups (2.5, 5, and 10 mg) (Figures 4B, 4C, and 4D) showed significantly higher numbers of regenerating myofibers (361.6 ± 84.39, 341.1 ± 57.21, 308.5 ± 84.33)

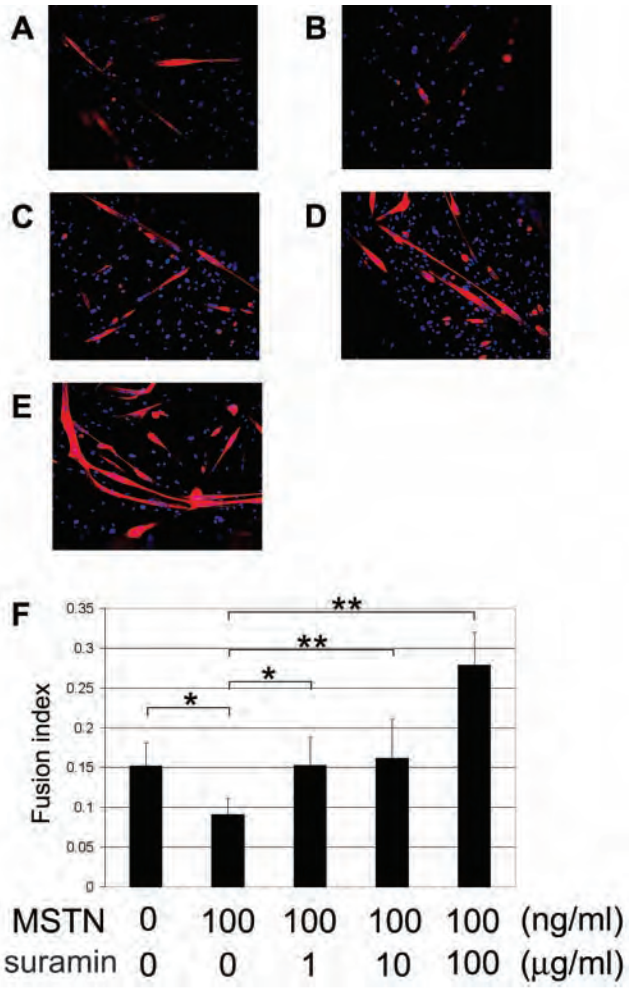

Figure 3. Immunocytochemical staining of muscle-derived stem cells (MDSCs) for fast myosin heavy chain at 3 days after incubation in 2% Dulbecco's modified Eagle's medium with different doses of suramin and myostatin (MSTN) (A, 0 μg/mL suramin and 0 ng/mL MSTN; B, 0 μg/mL suramin and 100 ng/mL MSTN; C, 1 μg/mL suramin and 100 ng/mL MSTN; D, 10 μg/mL suramin and 100 ng/mL MSTN; and E, 100 μg/mL suramin and 100 ng/mL MSTN). Myotubes are shown in red and nuclei are in blue (original magnification, ×200). F, comparison of fusion index of MDSC differentiation. *$P < .05$, **$P < .01$.

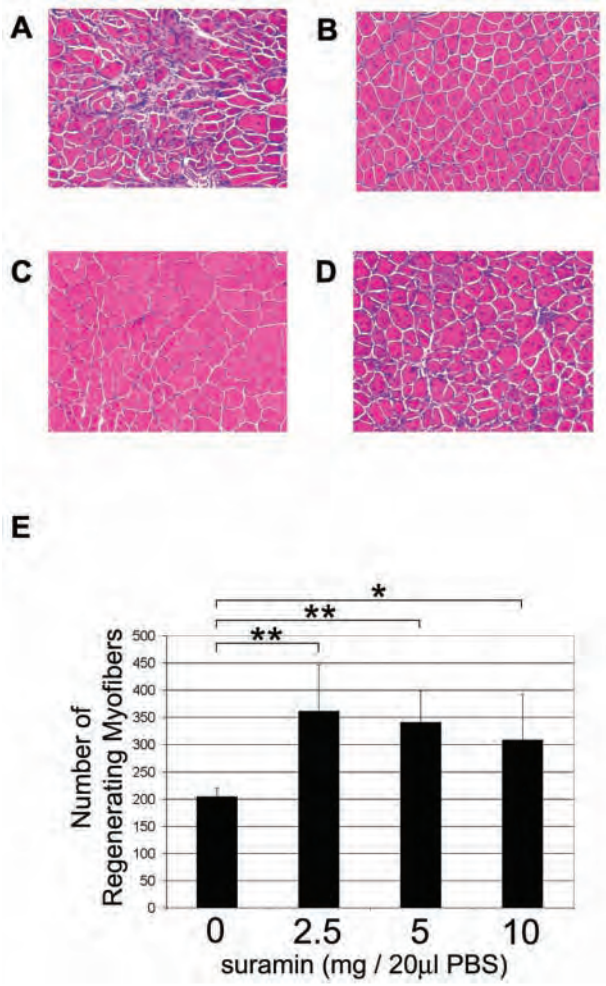

Figure 4. Histologic evaluation of muscle regeneration at four weeks after contusion injury by hematoxylin and eosin staining of tibialis anterior muscle treated with different concentrations of suramin (A, 0 mg/20 μL phosphate-buffered saline [PBS]; B, 2.5 mg/20 μL PBS; C, 5 mg/20 μL PBS; and D, 10 mg/20 μL PBS) injected at 2 weeks after injury. Regenerating myofibers were defined by centronucleated myofibers (original magnification, ×100). E, quantification of the number of regenerating myofibers treated with various concentrations of suramin. *$P < .05$, **$P < .01$.

compared with the control group (0 mg of suramin) (Figure 4A) (204.6 ± 15.27).

Effect of Suramin Therapy on Muscle Fibrosis After Contusion Injury. After Masson trichrome staining, the area of fibrotic scar tissue was evaluated and compared among the groups (Figure 5E). All the suramin-treated groups (2.5, 5, and 10 mg) (Figures 5B, 5C, and 5D) showed significantly less fibrotic area (6.856 ± 2.588, 7.677 ± 2.897, 8.993 ± 2.980) compared with the untreated control group (0 mg of suramin) (Figure 5A) (19.109 ± 3.215).

Suramin Injection Downregulated MSTN Expression in Injured Muscle. Immunohistochemical staining was performed to detect MSTN expression in the contusion-injured TA muscles. The MSTN-positive areas were measured and compared between the various treatment groups (Figure 6C). The muscle treated with 2.5-mg suramin injection 2 weeks after injury showed significantly less MSTN expression (4466.7 ± 7306.1) when compared with the untreated control (0 mg suramin) (27 830.2 ± 23 206.3) (Figures 6A and 6B).

Suramin Injection Improved Muscle Strength After Contusion Injury. The results of the physiologic evaluations are shown in Table 1. The control group (0 mg of suramin) and all the suramin-treatment groups, with the exception of the 2.5-mg suramin-treatment group, showed significantly less specific peak force (twitch and tetanic) when compared with the normal noninjured group. On the other hand, the muscles treated with 2.5 mg of suramin

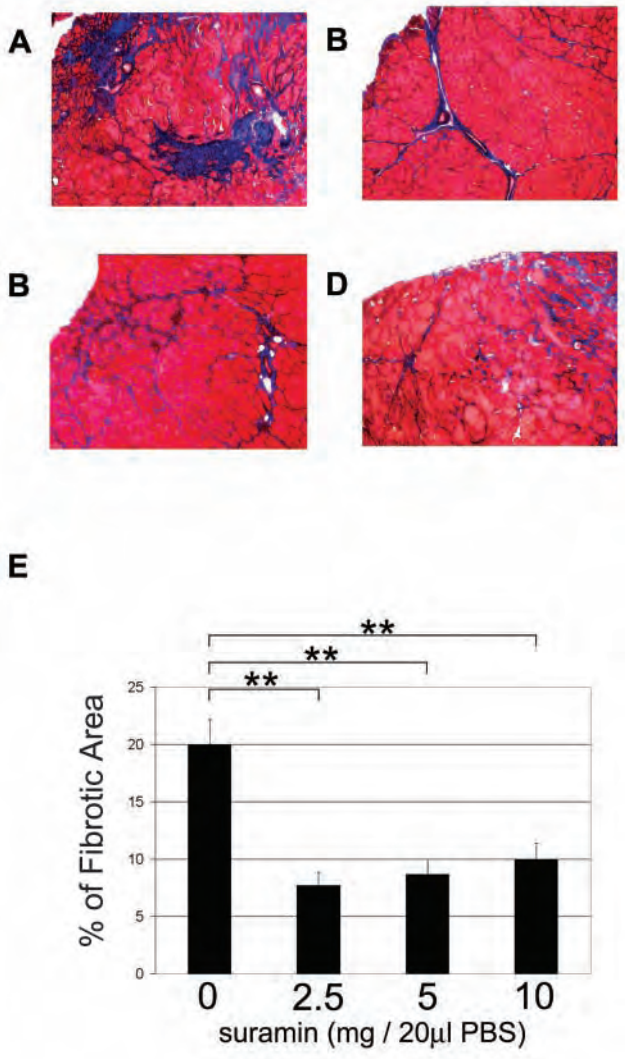

Figure 5. Histologic evaluation of the formation of scar tissue at 4 weeks after contusion injury by Masson trichrome staining of tibialis anterior muscle treated with different concentrations of suramin (A, 0 mg/20 µL phosphate-buffered saline [PBS]; B, 2.5 mg/20 µL PBS; C, 5 mg/20 µL PBS; and D, 10 mg/20 µL PBS) injected at 2 weeks after injury. Scar tissues are shown in blue and muscles are in red (original magnification, ×100). E, quantification of the scar tissue area in tibialis anterior muscle treated with various concentrations of suramin. $^{**}P < .01$.

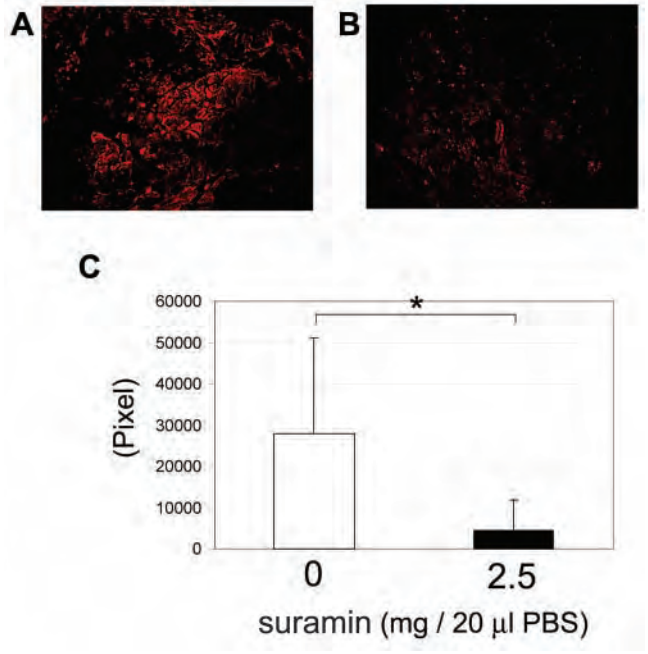

Figure 6. Immunohistochemical staining for MSTN at 2 days after suramin injection 2 weeks after injury (A, 0 mg suramin/20 µL phosphate-buffered saline [PBS]; B, 2.5 mg suramin/20 µL PBS). C, quantification of the total MSTN-positive area treated with suramin. $^{*}P < .05$.

TABLE 1
Results of Specific Peak Twitch and Titanic Force

Group	Specific Peak Twitch Force (N/cm^2)	Specific Peak Titanic Force (N/cm^2)
Normal (noninjured)	7.728 ± 0.312^a	23.272 ± 1.334^a
0 mg suramin	4.431 ± 0.903	13.797 ± 2.136
2.5 mg suramin	6.305 ± 1.239^b	21.055 ± 2.662^a
5 mg suramin	5.014 ± 0.337	15.131 ± 0.830
10 mg suramin	4.993 ± 1.074	15.085 ± 2.792

$^a P < .01$ compared with 0, 5, and 10 mg suramin groups.
$^b P < .05$ compared with 0 mg suramin group.

showed significantly greater specific peak force (twitch and tetanic) than the untreated control group. There was no significant difference between injured muscle that had been treated with 2.5 mg of suramin and normal noninjured muscle. In addition, the muscles injected with 2.5 mg of suramin also showed significantly greater specific peak tetanic force than the other suramin-treated groups (5 and 10 mg) and the control group (0 mg of suramin).

DISCUSSION

The aim of this study was to evaluate the effect of suramin on myogenic cell differentiation and muscle healing after contusion injury. We hypothesized that suramin treatment would lead to better biological healing by both stimulating muscle regeneration and preventing fibrosis in contused muscle. Histologically, the muscle treated with suramin 2 weeks after injury showed more regenerating myofibers and less fibrotic scar formation when compared with the control group (0 mg of suramin) at 4 weeks after contusion injury. Furthermore, suramin treatment also showed an increase in muscle strength compared with the untreated control group.

The muscle contusion injuries we modeled in this study are among the most common muscle injuries encountered in contact sports and by military personnel. More than

90% of muscle injuries are caused either by contusion or by excessive strain of the muscle.[3,14] Although contusion injury is capable of healing, incomplete functional recovery often occurs, depending on the severity of the initial trauma. Skeletal muscle has a great regenerative potential,[†] largely attributed to the activation of muscle progenitor cells and their fusion into mature multinucleated myofibers[22,40,41]; however, scar tissue formation occurs simultaneously and likely competes with muscle regeneration during the muscle-healing process.[12,26,32]

We have investigated the effect of some treatments, such as suture or immobilization, which are normally used in the clinic for muscle injury, by using animal models. One study showed that immobilization after muscle laceration had no significant effect on fibrosis reduction. Suture repair promoted better healing of the injured muscle and prevented the development of fibrosis at the deep-tissue level, but not superficially.[33]

Suramin, which is a polysulphonated naphthylurea, was designed as an antiparasitic drug[18] and is used for the treatment of human sleeping sickness, onchocerciasis, and other diseases caused by trypanosomes and other worms. In addition, it is under investigation as a treatment for some malignancies such as prostate, adrenal cortex, lymphoma, breast, and colon cancers[49] and for human immunodeficiency virus–1.[29] The major systemic side effects of suramin are malaise, neuropathy,[2,7] mineral corticoid insufficiency,[27] and corneal deposits[19]; occasionally neutropenia,[9] thrombocytopenia,[47] and renal failure[42] have also been observed. The toxicity of suramin when injected intramuscularly, as we did in our animal model, has not yet been determined. Although we observed no adverse effects in the mice injected with up to 10 mg of suramin in this study, we did not specifically test to evaluate side effects. Suramin is known as a heparin analog that can bind to heparin-binding proteins and inhibit the effect of growth factors by competitively binding to growth factor receptors.[43,49] Transforming growth factor-β1, -β2, and -β3; platelet-derived growth factor (PDGF) A and B; and epidermal growth factor are growth factors that are known to be inhibited by suramin. Among them, TGF-β1 and -β2 and PDGF A and B are known to have the potential to promote fibroblast proliferation.[44,46]

In our in vivo study using the muscle-contusion model, direct injection of suramin at 2 weeks after muscle injury demonstrated a significant reduction of fibrous tissue formation when compared with the untreated control (0 mg of suramin). We found that suramin treatment led to a beneficial effect in contused muscle, as was previously seen in suramin-treated lacerations and strains[5,6] as well as when using other antifibrotic agents such as decorin and interferon gamma.[12,13] Moreover, our in vivo results indicate that 2.5 mg of suramin was the optimal dosage for the treatment of muscle contusions as there was no significant difference observed when higher dosages (5 and 10 mg) of suramin was injected into the injury site. Our previous work with the laceration and strain models compared doses that were different than the current study and we have difficulty making direct comparisons between the current study and the former studies. In the laceration study, we compared suramin doses at 0.25, 1.0, and 2.5 mg and found that the 2.5-mg dose was optimal.[6] In the strain model study, we compared doses at 0.25, 1.0, and 5 mg and found that the 5-mg dose was optimal.[5] Because we did not use a 2.5-mg dose in the strain study and did not use a 5-mg dose in the laceration study, we cannot definitively say that the 2.5-mg dosage would be optimal for these types of injuries, although it is strongly inferred.

This study also showed that suramin enhanced muscle regeneration when it was injected directly into the muscle 2 weeks after receiving a contusion injury. We found many regenerating myofibers at the injury site, usually sequestered by a large amount of fibrotic tissue. All the suramin-treated groups showed a significant increase in the number of regenerating myofibers at 4 weeks after receiving the injury.

Functional recovery after muscle injury is the most important variable determining the likelihood for clinical translation of this therapy in the treatment of skeletal muscle injury. As was observed with other antifibrotic agents (decorin and interferon gamma),[12,13] suramin also appears to have a beneficial effect on the physiologic recovery of skeletal muscle. Our results showed that there was no significant difference in the specific peak twitch and tetanic forces between normal noninjured muscle and contused 2.5-mg suramin-treated muscle. These results strongly indicate that the injection of 2.5 mg of suramin at 2 weeks after contusion injury can improve muscle strength and promote functional recovery after muscle injury.

Taken together, our in vitro study about the effect of suramin on myogenic cell differentiation provides insight as to the underlying mechanism by which suramin enhances muscle regeneration in vivo. Our in vitro results showed 25 μg/mL of suramin can significantly enhance C2C12 differentiation at 4 days after incubation and, in addition, suramin treatment leads to significant increases in the fusion index in a dose-dependent manner. Further, we observed an even more prominent dose-dependent effect on MDSC differentiation 3 days after initiating the incubation with suramin. Moreover, suramin also showed a neutralizing effect on MSTN, which inhibits differentiation of MDSCs in a dose-dependent manner.

We found that suramin stimulates myogenic differentiation in vitro. It is consistent with our in vivo results, showing that suramin is capable of enhancing muscle regeneration and improving muscle healing after muscle injury. Furthermore, as expected, we observed that MSTN significantly inhibits the myogenic differentiation of MDSCs; however, in the presence of suramin, the inhibitory effect of MSTN on myogenic differentiation was attenuated in a dose-dependent manner. These results suggest that suramin can neutralize MSTN activity. Therefore, we hypothesize that when myoblasts and MDSCs are treated with suramin, suramin stimulates myogenic differentiation by neutralizing the effect of endogenous MSTN. A similar neutralizing effect on MSTN has been seen when cells were treated with decorin, a TGF-β1 blocker.[48]

[†]References 4, 14, 16, 21, 26, 28, 33, 45

On the basis of these results, we further investigated whether suramin exerts a beneficial effect in the injured muscle through regulating MSTN expression. Not surprisingly, it was revealed that treatment with suramin significantly decreased MSTN expression in the injured muscle. Our previous study showed that MSTN can act with TGF-β1 to magnify fibrosis cascades in injured muscles.[48] Taken together, it may suggest that suramin administration effectively leads to enhanced muscle regeneration and reduced fibrosis after muscle injury via downregulation of endogenous MSTN.

This is the first study conducted to evaluate the effects that suramin would have on a contusion injury. It is also the first study to demonstrate that one of the mechanisms by which suramin could enhance skeletal muscle healing was by the downregulation of MSTN. Our results showed suramin can enhance myogenic cell differentiation and neutralize the effects of MSTN, which downregulates MDSC differentiation. Moreover, these in vitro results may reveal a possible mechanism by which suramin directly enhances muscle regeneration after muscle injury. Future studies should investigate further the mechanism by which suramin appears to stimulate myogenic differentiation and promotes muscle healing via MSTN regulation.

In summary, we demonstrated that the direct injection of suramin at 2 weeks after contusion injury can effectively reduce fibrotic scar formation and enhance muscle regeneration 4 weeks after injury. Physiologic evaluation also showed that suramin can enhance muscle functional recovery after contusion injury. Our in vitro study demonstrated that culturing either C2C12 myoblasts or MDSCs with suramin led to a significant increase in the fusion index of the cells. Moreover, with the addition of both suramin and MSTN in cell cultures, suramin was able to counteract MSTN's biological activity, thereby rescuing MSTN-inhibited myogenic differentiation of MDSCs.

The greatest advantage of using suramin is that this drug has already been approved by the FDA.[34,39] Future studies should consider the use of this agent for off-label use in the treatment of skeletal muscle injuries. Our findings could contribute to the development of progressive therapies for treating skeletal muscle injuries.

ACKNOWLEDGMENT

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Angiotensin II Receptor Blockade Administered After Injury Improves Muscle Regeneration and Decreases Fibrosis in Normal Skeletal Muscle

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Angiotensin II Receptor Blockade Administered After Injury Improves Muscle Regeneration and Decreases Fibrosis in Normal Skeletal Muscle

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Background: Several therapeutic agents have been shown to inhibit fibrosis and improve regeneration after injury in skeletal muscle by antagonizing transforming growth factor-β1. Angiotensin receptor blockers have been shown to have a similar effect on transforming growth factor-β1 in a variety of tissues.

Hypothesis: Systemic treatment of animals after injury of skeletal muscle with an angiotensin receptor blocker may decrease fibrosis and improve regeneration, mainly through transforming growth factor-β1 blockade, and can be used to improve skeletal muscle healing after injury.

Study Design: Controlled laboratory study.

Methods: Forty mice underwent bilateral partial gastrocnemius lacerations. Mice were assigned randomly to a control group (tap water), a low-dose angiotensin receptor blocker group (losartan, 0.05 mg/mL), or a high-dose angiotensin receptor blocker group (0.5 mg/mL). The medication was dissolved in tap water and administered enterally. Mice were sacrificed 3 or 5 weeks after injury, and the lacerated muscles were examined histologically for muscle regeneration and fibrosis.

Results: Compared with control mice at 3 and 5 weeks, angiotensin receptor blocker–treated mice exhibited a histologic dose-dependent improvement in muscle regeneration and a measurable reduction in fibrous tissue formation within the area of injury.

Conclusion: By modulating the response to local and systemic angiotensin II, angiotensin receptor blocker therapy significantly reduced fibrosis and led to an increase in the number of regenerating myofibers in acutely injured skeletal muscle. The clinical implications for this application of angiotensin receptor blockers are potentially far-reaching and include not only sports- and military-related injuries, but also diseases such as the muscular dystrophies and trauma- and surgery-related injury.

Clinical Relevance: Angiotensin receptor blockers may provide a safe, clinically available treatment for improving healing after skeletal muscle injury.

Keywords: fibrosis; angiotensin receptor blocker; TGF-β; muscle injury; muscle regeneration

Skeletal muscle injuries are among the most common complaints of patients seen by general medical physicians and also account for a large majority of patients seen in orthopaedic clinics.[2] Investigations have demonstrated that the natural history of muscle injury proceeds through a highly coordinated sequence of steps. Unfortunately, the regenerative capacity of injured skeletal muscle is limited; fibrotic tissue is a common end result that predisposes the muscle to recurrent injury and limits recovery of function.[16] Clinical experience reveals a high recurrence rate of skeletal muscle strain injuries among athletes, approaching 30% in some professional-level athletes.[32] Advancements in the identification of molecular events and cellular transformations after muscle injury have flourished; however, the clinical treatment of this common condition still relies on conventional therapies of rest, ice, and anti-inflammatory medications; these are of limited efficacy in preventing or

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treating posttraumatic muscle fibrosis and in reducing the rate of reinjury.[1,18,33]

In our laboratory, we have investigated several biological agents that have proved to be of some benefit in altering the natural course of muscle injury. Specifically, we have focused our recent efforts on agents that inhibit muscle fibrosis via inhibition of transforming growth factor-β1 (TGF-β1), a key cytokine in the fibrotic signaling pathway in skeletal muscle.[21] Using decorin, suramin, relaxin, and gamma interferon, we have demonstrated that these therapies can decrease fibrosis and increase regeneration after skeletal muscle injury.[9,13,14,22,24] However, their use clinically is hampered by relatively severe side-effect profiles, lack of oral dosing formulations, and in some cases, lack of Food and Drug Administration (FDA) approval for use in humans.

Fibrosis is a pathologic process that is not unique to the skeletal muscle system. Observations have linked pathologic fibrosis in various organ systems to the local effects of a naturally occurring molecule, angiotensin II, an end product of the blood pressure–regulating renin-angiotensin system. The modulation of angiotensin II with angiotensin-converting enzyme inhibitors or angiotensin II receptor blockers has demonstrated decreased fibrosis and improved function in liver, kidney, and lung tissue.[23,27,28,30] Injured cardiac muscle in disease entities such as congestive heart failure also demonstrates dysfunction related to fibrosis. Myocardium exposed to decreased levels of angiotensin II, either through the use of angiotensin-converting enzyme inhibitors or angiotensin receptor blockers (ARB), has also demonstrated measurably improved function.[15,31] Investigators have also observed a relationship between the modulation of angiotensin II and skeletal muscle. Patients treated with angiotensin-modulating medications for hypertension also displayed the unexpected side effect of decreased rates of muscle wasting and a reduction in the relative amount of adipose tissue within their musculature.[25] Moreover, elite athletes, particularly those in endurance sports, have also demonstrated findings consistent with inherent differences in their body's metabolism of angiotensin II, with decreased exposure resulting in improved skeletal muscle function.[25] We have observed an improvement in skeletal muscle regeneration postinjury after treatment with angiotensin blockade; this is a finding that, to our knowledge, had not been reported previously.[4] Elegant experiments have subsequently been reported that elucidate the mechanism by which angiotensin II receptor blockade modulates TGF-β1, which has also been implicated in the prevention of muscle regeneration in murine models of chronic myopathic disease.[10] The hypothesis of this study is that systemic delivery of an angiotensin II receptor blocker can enhance muscle regeneration and decrease fibrosis in a laceration model of normal skeletal muscle injury. This therapy may be an effective treatment when instituted postinjury, which is a clinically relevant scenario for physicians treating muscle injuries.

MATERIALS AND METHODS

In Vitro

The role of angiotensin II regarding myoblast and fibroblast proliferation was investigated using a series of standard proliferation experiments. National Institutes of Health (NIH) 3T3 cells (fibroblast cell line) and C2C12 myoblasts, purchased from American Type Culture Collection (ATCC) (Rockville, Maryland), along with primary muscle fibroblast isolates (PP2 cells) obtained through the previously published preplating technique, were chosen for analysis.[29] These cells were cultured with Dulbecco's modified Eagle's medium (DMEM) (Invitrogen, Carlsbad, California) containing 10% fetal bovine serum, 10% horse serum, 0.5% chicken embryo extract, and 1% penicillin/streptomycin. All cells were cultured at 37°C in 5% CO_2. National Institutes of Health 3T3, C2C12, and PP2 cells were seeded onto different 6-well plates. Cells were cultured with medium (described above) and 0, 10^{-8}, 10^{-6}, or 10^{-4} mol/L of human angiotensin II (Sigma Chemical, St Louis, Missouri). Cell counts were performed at 24, 48, and 96 hours using a standard cell-counting chamber.

The supernatant solution from the PP2 cell culture was extracted after 24 and 48 hours of exposure to the various concentrations of angiotensin II. An enzyme-linked immunosorbent assay (ELISA) was performed in accordance with the manufacturer's recommendations (Sigma Chemical) to determine the level of TGF-β1 in the solution. National Institutes of Health 3T3 cells were harvested 48 hours after incubation with or without angiotensin II, and a western blot was performed. After lysation, the samples were separated by 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes used for immunostaining. Mouse anti-TGF-β1 type I antibodies (Sigma Chemical) were then applied. The horseradish peroxidase-conjugated secondary antibodies (Pierce, Rockford, Illnois) were developed using SuperSignal West Pico Chemiluminescent substrate (Pierce), and positive bands were visualized on radiographic film. All results were analyzed using Northern Eclipse software v.6.0 (Empix Imaging, Mississauga, Ontario, Canada).

Animal Model

Forty immunocompetent mice (C57Bl/6J; Jackson Laboratory, Bar Harbor, Maine) were used for the histologic analysis. The animals were housed in cages and fed with commercial pellets. The policies and procedures of the animal laboratory were in accordance with those detailed by the US Department of Health and Human Services and the Animal Research Care Committee of the authors' institution (Protocol #25-03). A previously reported muscle laceration injury model was employed for these experiments, the healing process of which is similar histologically to strain injury models, which account for the most common clinical pattern of injury.[9,14,17,19,21] The mice were anesthetized with 16 μL of ketamine and 8 μL of xylazine in 25 μL of phosphate-buffered saline by intraperitoneal injection. For histologic analysis, lacerations were performed bilaterally on the gastrocnemius muscle through 50% of its width and 100% of its thickness at 60% of its length from its insertion. All mice were randomly assigned to 1 of 3 groups: (i) a control group, (ii) a low-dose ARB group, or (iii) a high-dose ARB group. The control group was fed tap water, while the low and high-dose ARB groups were fed commercially available losartan (Cozaar; Merck & Co, West Point,

Pennsylvania) dissolved in tap water at concentrations of 0.05 g/L, and 0.5 g/L, respectively. These doses were calculated based on the average fluid intake of a population of mice with similar demographic backgrounds to those tested in this study, and are well below reported toxicity levels. All animals were caged separately and allowed access to the water or ARB solutions ad libitum from the time of injury to the time of sacrifice. The animals were not pretreated in any way. The average daily fluid intake of each animal was monitored and recorded. Mice were sacrificed at 3 and 5 weeks. The injured muscles were isolated, mounted, and snap-frozen in liquid nitrogen-cooled 2-methylbutane. Samples were serially sectioned at 10 μm with a cryostat for histologic analysis.

Angiotensin Receptors in Skeletal Muscle

Immunohistochemistry was used to evaluate the presence and distribution of angiotensin II receptors in injured and uninjured skeletal muscle. Commercially available antibodies (Abcam Inc, Cambridge, Massachusetts) were used to localize angiotensin II receptors in skeletal muscle. The same sections were also stained with collagen IV antibodies (Biodesign International, Saco, Maine), which stain the extracellular matrix (basal lamina) of muscle fibers, and 4′-6-diamidino-2-phenylindole (DAPI) to delineate nuclear location. A blinded observer performed all analyses.

Muscle Fibrosis

To detect the amount of fibrosis in the area of injury, sections from each limb of each animal were washed in deionized water and stained with a Masson Modified IMEB Trichrome Stain Kit (IMEB Inc, San Marcos, California) according to the manufacturer's specifications. This technique, which stains nuclei black, muscle red, and collagen blue, was previously validated through immunohistochemistry as an accurate technique for evaluating fibrous tissue within skeletal muscle.[6,13,20] Five randomly selected 200× high-powered image fields within the injured area for each limb were obtained using a Nikon Eclipse 800 light microscope (Tokyo, Japan) fitted with a Spot-RT digital camera (Diagnostic Instruments, Sterling Heights, Michigan). Images were analyzed using Northern Eclipse image analysis software (Empix Imaging) to measure the percent area of collagen (blue staining tissue) within the injury zone. Color threshold levels within the software program were set to isolate the blue staining regions and calculate the area of that region that corresponded to the area of fibrosis. This value was expressed as a percentage of the entire cross-sectional area of the muscle section. A blinded observer performed all analyses.

Muscle Regeneration

Muscle sections were stained with hematoxylin and eosin. Muscle regeneration was assessed by counting the number of centronucleated myofibers. Five 200× high-powered fields throughout the injury zone were analyzed from each muscle, and the average number of regenerating myofibers per field was calculated. A blinded observer performed all analyses.

TABLE 1
Varying Concentrations of Angiotensin II With Myoblast (C2C12) and Fibroblast (3T3, PP2) Cell Lines Did Not Affect Proliferation at 24, 48, or 72 Hours[a]

Concentrations, M	24 h	48 h	72 h
C2C12			
0	125	114	160
10^{-8}	145	135	150
10^{-6}	130	140	200
10^{-4}	110	135	140
PP2			
0	25	25.5	40
10^{-8}	30	21.5	65.5
10^{-6}	25	24.5	37.5
10^{-4}	22	24.5	34.5
3T3			
0	102.5	570	1205
10^{-8}	111.5	611.5	1144
10^{-6}	124.25	612.5	1158
10^{-4}	141.875	139.8	112.7

[a]Angiotensin II concentration is expressed in molar amounts, and cell counts are expressed as thousands of cells ($\times 10^3$ cells).

Statistical Methods

Comparisons of muscle fibrotic area and number of regenerating myofibers data were performed by means of a 2-way analysis of variance (ANOVA) and Tukey's post-hoc test using SPSS software, version 16.0 (SPSS Inc, Chicago, Illinois). Immunohistochemical data are presented and compared qualitatively.

RESULTS

In Vitro

Angiotensin II does not affect cell proliferation. Angiotensin II appeared to have no effect on C2C12 (myoblast cell line), NIH 3T3 (fibroblastic cell line), or PP2 (fibroblast cell line) proliferation in vitro compared with controls at 24, 48, or 72 hours of culture (Table 1).

Angiotensin II increases the production of TGFβ-1 in fibroblasts. Fibroblasts isolated from normal skeletal muscle (PP2 cells) appear to up-regulate the expression of TGF-β1 detected by ELISA in the supernatant fluid when cultured with angiotensin II in a dose-independent manner at both 24 and 48 hours. National Institutes of Health 3T3 fibroblasts also appear to respond to the presence of angiotensin II by expressing TGF-β1, as detected by western blotting (data not shown).

In Vivo

Angiotensin II receptors in skeletal muscle. Immunohistochemistry demonstrated the presence of angiotensin II receptors in both normal and injured skeletal muscle. The

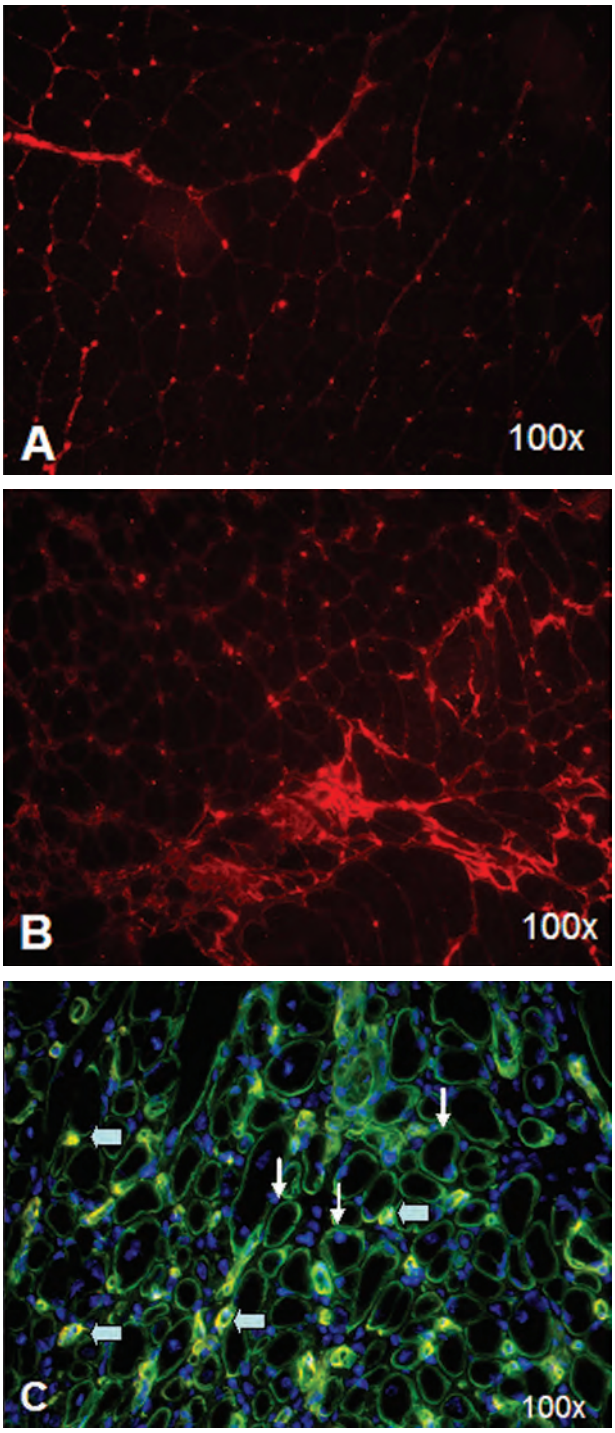

Figure 1. Immunohistochemistry demonstrating increased expression of the angiotensin II receptor (red) in injured (B) versus noninjured (A) skeletal muscle. C, the spatial relationships between collagen IV (green), angiotensin II receptor (red), nuclei (blue), and colocalization (yellow) are shown. The angiotensin II receptor distribution (thick arrows) appears more frequently within the extracellular matrix and sarcolemma of muscle fibers than within the myofibers themselves (thin arrows).

distribution of the receptors in normal muscle appeared to be sparse and evenly distributed along the cell membrane. After injury, the expression of angiotensin II receptors appeared elevated, particularly within the zone of injury. The expression of these receptors appeared to be greater within the cellular structure of the extracellular matrix, rather than on the membrane of the muscle fibers (Figure 1).

Animal Model

All mice used in this experiment were male. No mice demonstrated any ill effects from the treatment regimen, and there were no observable differences in the activity level in any of the groups; no deaths were attributable to the therapeutic intervention. The age of all animals at the beginning of the study ranged from 4 to 6 weeks, and the average weight was 21.4 ± 2.3 g. There was no significant difference in the weight of the animals in the experimental or control groups at any time point. The average dose of ARB received by each animal in the low-dose group and the high-dose group was approximately 0.027 ± 0.015 g/kg/d and 0.298 ± 0.20 g/kg/d, respectively.

Skeletal Muscle Regeneration

At 3 weeks, the average number of regenerating myofibers within the zone of injury (as identified by centrally located nuclei) was 56 ± 14 fibers per 200× high-powered field (n = 4) in the control group, 93 ± 25 (n = 8) in the low-dose group, and 95 ± 32 (n = 8) in the high-dose group (Figure 2). In injured animals treated for 5 weeks, a statistically significant increase in regenerating myofibers was observed. This increase was 45 ± 16 fibers per 200× high-powered field (n = 5) in the control group, 86 ± 19 (n = 8; $P < .01$ vs control group) in the low-dose group, and 103 ± 21 (n = 7; $P < .01$ vs control group) in the high-dose group. At both 3 and 5 weeks, the authors observed a trend toward increasingly greater numbers of regenerating myofibers as both the dose and duration of treatment increased (Figure 2).

Skeletal Muscle Fibrosis

Three weeks after muscle injury, without benefit of treatment, the control group had an area of fibrosis within the zone of injury equal to 34% ± 17% (n = 4). The low-dose treatment group had significantly less fibrosis within the zone of injury (16% ± 9%; n = 8; $P < .05$) when compared with the control group. The high-dose treatment group also had significantly less fibrosis (7% ± 4%; n = 8; $P < .01$ vs control) when compared with the control group. At 5 weeks after injury, the control group, low-dose group, and high-dose group had fibrosis areas of 45% ± 22% (n = 5), 19% ± 9% (n = 8; $P < .01$ vs control), and 14% ± 11% (n = 7; $P < .01$ vs control), respectively, demonstrating a similar trend toward an attenuation of fibrosis mediated by the administration of losartan (Figure 3).

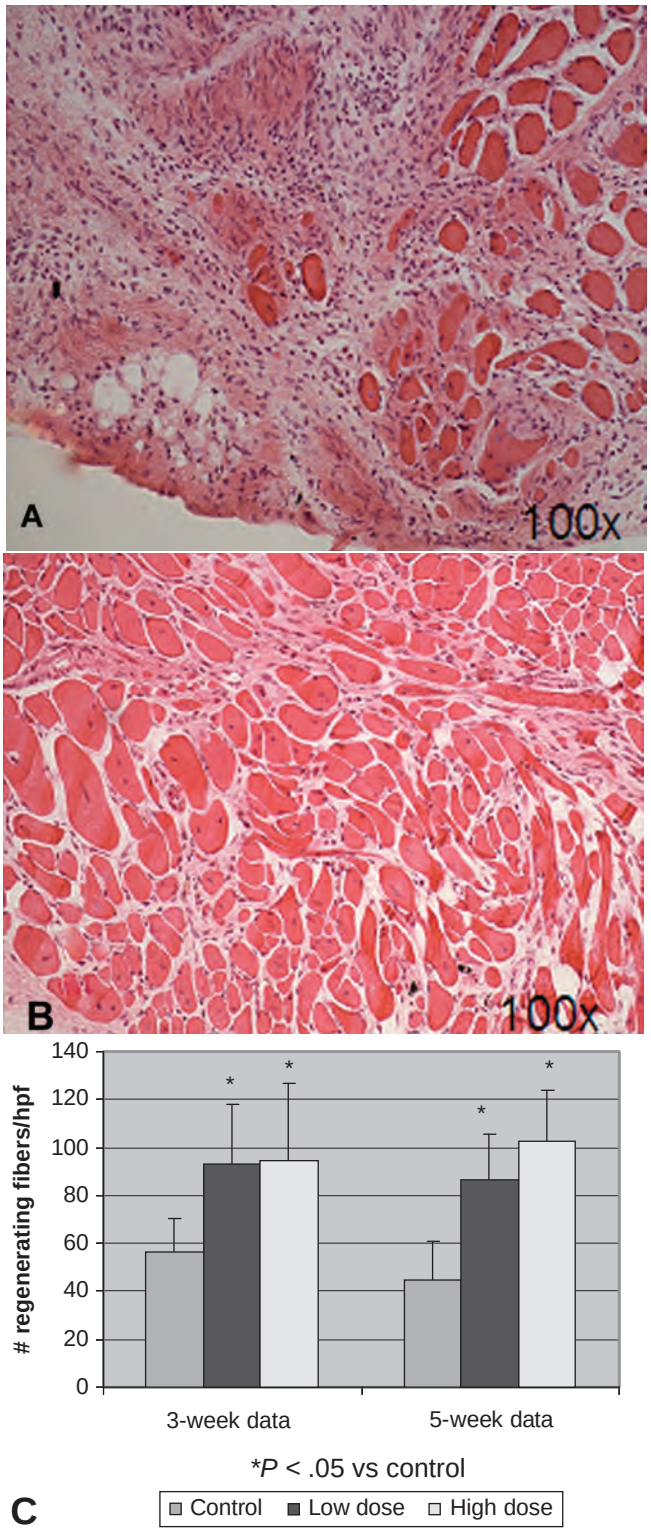

Figure 2. Characteristic hematoxylin-and-eosin–stained sections taken 5 weeks after laceration injury of the gastrocnemius muscle. Note the greater number of regenerating myofibers (recognized by their centrally located nuclei) in the high-dose angiotensin receptor blocker (ARB)–treated group (B) compared with the control group (A). The graph (C) depicts an increase in the number of regenerating myofibers in ARB-treated animals compared with controls. Error bars represent standard deviation. hpf, high-powered field.

DISCUSSION

The impetus for investigating the antifibrotic properties of ARBs in muscle healing arises from prior research on angiotensin II, a known vasoconstrictor and target of many antihypertensive medications, demonstrating its deleterious effects on smooth and myocardial muscle tissues after injurious insults. The role of angiotensin II in muscle fibrosis after injury is well documented in the cardiac literature, in which antagonism of angiotensin II with ARBs is noted to significantly improve cardiac contractility and cardiac output.[15] Moreover, histologic analysis indicates that the mechanism of cardiac improvement is related to decreased fibrosis and enhanced regeneration. The effect of ARBs on skeletal muscle healing, however, remains largely unstudied until recently.

The natural history of skeletal muscle injury is that of residual fibrosis within regenerate tissues that predisposes muscle to reinjury and reduced functionality, thereby constituting a significant clinical burden. After injury, the initial phase of myofiber regeneration wanes over time as it is replaced by fibrous tissue. A reduction of fibrous tissue and the improvement of muscle regeneration correlate with enhanced function.

Several investigations have observed this by treating injured skeletal muscle with antifibrotic agents that directly antagonize TGF-β1, and the digestion of existing fibrous tissue deposits appears to enhance myofiber regeneration.[5,9,13,14,23,24] Research on the pathogenesis of fibrosis further reveals that local levels of TGF-β1 during the fibrotic phase are elevated along the zone of injury[21] as a potential result of angiotensin II.[10]

In a mechanism consistent with these studies, we show through ELISA and western blot assays that fibroblasts increase TGF-β1 production when they are exposed to angiotensin II. We also show that angiotensin II receptors are more densely distributed within the extracellular matrix of the zone of injury, suggesting that angiotensin II receptor upregulation is intimately related to the deposition of fibrous tissue. Our results further demonstrate that inhibiting angiotensin II with an ARB diminishes post-traumatic fibrosis.

We have previously focused our attention on muscle injury models such as ischemia, contusion, strain, and cardiotoxin injection. While skeletal muscle laceration is a less frequently observed injury pattern, its histopathology is nearly identical to the more commonly observed strain injury patterns. Our purpose in choosing the laceration model in this experiment was 2-fold. First, it represents a reproducible model for evaluating skeletal muscle injury, which is a challenge with the more common strain-type injury model because of difficulty in accurately reproducing and delivering the same magnitude of injury between samples. Second, the laceration model establishes a "worst-case scenario" in which myofibers are completely transected. Observations of any treatment effect in this worst-case injury model would likely be of equal or, perhaps, greater benefit in less severe and more commonly encountered problems like strain injuries. In addition, the animals were not pretreated with ARB before injury, as this more closely resembles clinically relevant scenarios in which muscle injuries are treated. While many potential

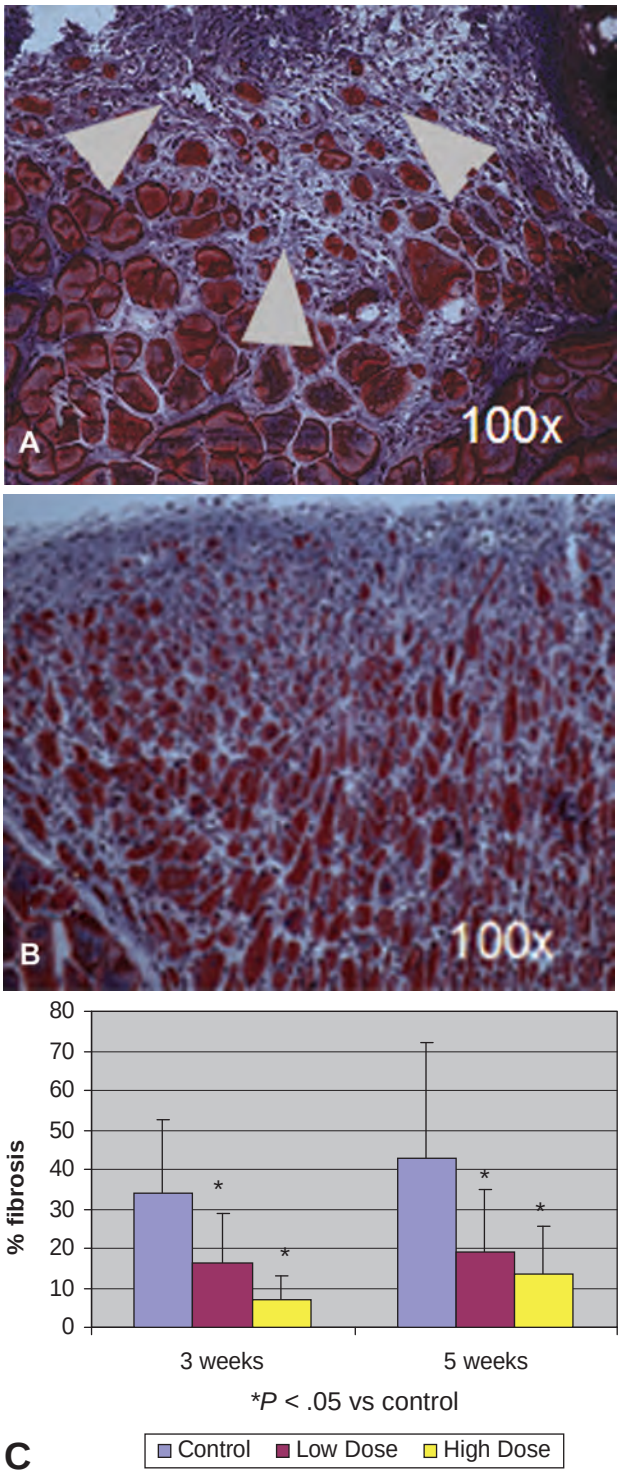

Figure 3. Masson's trichrome (collagen: blue, myofibers: red, nuclei: black) staining of sections taken 5 weeks after laceration injury of the gastrocnemius. Note the dense blue staining fibrous tissue in the control group (A) (arrowheads) compared with the high-dose angiotensin receptor blocker (ARB)–treated group (B). Both pictures are taken at 100× magnification. The graph (C) depicts a decrease in the amount of fibrosis in ARB-treated animals compared with controls. Error bars represent standard deviation.

problems exist with the use of an antihypertensive medication, all doses administered to the animals in this study were below the corresponding human dose equivalent for the antihypertensive effect of losartan. Accordingly, there were no ill effects observed in any of the animals.

Treating skeletal muscle-injured mice with an ARB for extended periods of time reduces the amount of fibrosis and enhances the number of regenerating myofibers in the zone of injury. This occurs in a duration-dependent manner, where medication administered for longer periods of time provided greater regenerative benefit. The observation of a positive regenerative effect after only 5 weeks of treatment, as opposed to other models where treatment was given for up to 9 months, is of notable significance. Prolonged treatment for acute skeletal muscle injuries would seem to be a less attractive and less pragmatic approach.

Similar success in reducing residual fibrosis and enhancing myofiber regeneration, as well as success in improving function, has been observed in animal models, albeit after treatment with potentially harmful compounds. As noted above, we have demonstrated that decorin, suramin, gamma-interferon, and relaxin can all decrease residual fibrosis, enhance myofiber regeneration, and improve function in animal models of skeletal injury.[9,13,14,22,24] However, these compounds are not commonly employed in clinical practice, and several of these are known to have relatively severe side effects that may preclude their use in the treatment of muscle injury.

While the clinical implications of identifying efficacious antifibrotic therapies are particularly important in the field of sports medicine, they apply to an even broader spectrum of orthopaedic patients. Athletes with muscle strains often struggle with lengthy recovery periods in rehabilitation that can limit their duration of play and predispose them to recurrent injuries that oftentimes are more severe.[26] For orthopaedic trauma patients, contusions and lacerations are markedly common, and for all patients undergoing elective and emergent procedures, many surgical incisions are essentially controlled lacerations of skeletal muscle. In each type of patient, the quality of regenerate skeletal muscle and the rate of recovery are, ultimately, compromised and delayed by fibrosis. For some patients, this can result in prolonged immobilization with greater risks for developing a deep venous thrombus or pneumonia. Because the clinical use of angiotensin receptor II inhibitors is not currently approved for orthopaedic surgery, clinical trials are necessary to further determine the effect of this class of medication on skeletal muscle healing, as well as on the functional outcomes, morbidity, and mortality associated with skeletal muscle injury and prolonged rehabilitation.

In light of the possibility that our work may pave the way for clinical trials on the effect of losartan as an antifibrotic therapy to promote optimal skeletal muscle healing, it is important to draw attention to potential adverse effects of angiotensin II receptor antagonists. The side effect profile of ARBs is surprisingly minimal compared with other antihypertensive medications; the only reported dose-related side effect is hypotension, while non-dose–related adverse outcomes include headaches, dizziness,

weakness, and fatigue.[3] This is particularly worth mentioning because these side effects can theoretically affect athletic performance on the field as well as patient performance in a rehabilitation program. Accordingly, clinicians and therapists must be vigilant in monitoring for such effects. While uncommon, there have also been reports of raised liver enzymes, cholestatic hepatitis, and pancreatitis with losartan.[7,8,23] Finally, while ARBs are more well known for their renoprotective effects, any diuretic generally carries the potential for acute renal insufficiency, which can be a particular concern that especially warrants mentioning in settings where patients may be dehydrated from athletic exertion or blood losses in postoperative and trauma settings.

In summary, the use of the ARB losartan, a commercially available, generally well-tolerated, safe, and widely used antihypertensive medication, has been demonstrated in this study to enhance muscle healing by reducing fibrosis and enhancing muscle regeneration in doses below those required for any antihypertensive effect. These findings may represent the most clinically appealing and potentially far-reaching antifibrotic therapy developed so far for otherwise healthy individuals who have sustained acute skeletal muscle injuries.

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ORIGINAL ARTICLE

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Interaction Between Macrophages, TGF-β1, and the COX-2 Pathway During the Inflammatory Phase of Skeletal Muscle Healing After Injury

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Inflammation, an important phase of skeletal muscle healing, largely involves macrophages, TGF-β1, and the COX-2 pathway. To improve our understanding of how these molecules interact during all phases of muscle healing, we examined their roles in muscle cells in vitro and in vivo. Initially, we found that depletion of macrophages in muscle tissue led to reduced muscle regeneration. Macrophages may influence healing by inducing the production of TGF-β1 and PGE_2 in different muscle cell types. We then found that the addition of TGF-β1 induced PGE_2 production in muscle cells, an effect probably mediated by COX-2 enzyme. It was also found that TGF-β1 enhanced macrophage infiltration in wild-type mice after muscle injury. However, this effect was not observed in COX-$2^{-/-}$ mice, suggesting that the effect of TGF-β1 on macrophage infiltration is mediated by the COX-2 pathway. Furthermore, we found that PGE_2 can inhibit the expression of TGF-β1. PGE_2 and TGF-β1 may be involved in a negative feedback loop balancing the level of fibrosis formation during skeletal muscle healing. In conclusion, our results suggest a complex regulatory mechanism of skeletal muscle healing. Macrophages, TGF-β1, and the COX-2 pathway products may regulate one another's levels and have profound influence on the whole muscle healing process.

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Tissue naturally responds to injury caused by trauma, microorganisms, toxins, or other causes through inflammation. Cytokines including histamine, bradykinin, serotonin, prostaglandins and others are released by damaged tissue and cause fluid leakage from blood vessels. These cytokines also attract white blood cells to infiltrate and migrate to the injury site to digest pathogens and dead/damaged cells, a process called phagocytosis. White blood cells and cytokines are of paramount importance for protection against infection and the invasion of foreign substances. Furthermore, they are involved with the regeneration of damaged tissues (Prisk and Huard, 2003; Tidball, 2005).

Skeletal muscle injury is a common type of injury associated with sports activities, high speed vehicle accidents, and military combat. In injured skeletal muscle, the inflammation response starts rapidly after trauma, and causes symptoms that include muscle pain, swelling, fever, and loss of mobility. To relieve these symptoms, many different non-steroidal anti-inflammatory drugs (NSAIDs) are used to block the cyclooxygenase (COX) enzymes and the products of the COX pathway, the prostaglandins. Early studies reported that NSAIDs had a favorable effect in quickly reducing muscle weakness and functional loss in the short term following muscle injury (Hasson et al., 1993; Obremsky et al., 1994; Mishra et al., 1995; Dudley et al., 1997). However, some studies reported either no effect or a detrimental effect on long term muscle strength recovery after prolonged NSAIDs treatment (Mishra et al., 1995; Almekinders, 1999). Recently, studies using COX-2 specific inhibitors also showed that the COX-2 pathway, which is mostly induced in pathological situations, is important in promoting muscle regeneration and reducing fibrosis formation (Bondesen et al., 2004; Shen et al., 2005). Furthermore, it is suggested that the prostaglandins, although the cause of uncomfortable symptoms, are actually beneficial in promoting skeletal muscle healing (Horsley and Pavlath, 2003; Pavlath and Horsley, 2003; Prisk and Huard, 2003).

Interestingly, it has been suggested that two other components of inflammation, macrophages and transforming growth factor-beta1 (TGF-β1), are related to the COX-2 pathway. Macrophages are an important source of COX-2 enzymes and prostaglandins during the inflammation phase

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(Graf et al., 1999; Lee et al., 2002). And, by some reports, the inhibition of the COX-2 pathway reduced the infiltration of macrophages to the injury site (Bondesen et al., 2004; Shen et al., 2005). TGF-β1, well known for its fibrotic effect, was also shown to be important in the inflammation phase as a result of its connection to the COX-2 pathway. TGF-β1 may increase the production of prostaglandin E_2 (PGE_2) through COX-2, and PGE_2 may inhibit fibroblast proliferation and collagen production to balance the fibrotic effect of TGF-β1 (Goldstein and Polgar, 1982; McAnulty et al., 1997; Keerthisingam et al., 2001). Furthermore, macrophages and TGF-β1 may also be connected to one another, as several studies have shown that macrophages may be an important source of TGF-β1 (Khalil et al., 1989, 1993; Wolff et al., 2004). However, little evidence of these interactions has been demonstrated in muscle cells during skeletal muscle injury.

To further the understanding of the healing mechanism of skeletal muscle injury and the role of inflammation in the muscle healing process, we examined the relationship between the COX-2 pathway, macrophages, and TGF-β1 in skeletal muscle inflammation. We found that these important components form a complex network wherein they appear to regulate each other. These interactions may not only modulate muscle inflammation, but also influence the regeneration and fibrosis phases of skeletal muscle healing.

Materials and Methods

Cell isolation and culturing

Myogenic precursor cells (MPCs) were isolated via a previously described preplate technique (Rando and Blau, 1994; Qu et al., 1998). Briefly, gastrocnemius muscles (GMs) were removed from 4-week-old C57BL/6J mice (Jackson Laboratories, Bar Harbor, ME), and minced with scissors. The minced tissues were enzymatically digested by sequential exposure to collagenase, dispase, and trypsin. The muscle cell extracts were then plated on collagen-coated flasks, and different populations were isolated by re-plating the extracts after different time intervals. The late plated population is usually composed of MPCs that have high myogenic potential when induced by low serum culture medium (Qu et al., 1998). Exudate macrophages were isolated according to the technique described before (Robertson et al., 1993). Briefly, mice were injected intraperitoneally with 30 ml thioglycollate broth (BD Biosciences, San Jose, CA). Three days later, the peritoneal macrophages were harvested by centrifugation. Macrophages and MPCs were used for in vitro experiments along with two other cell lines, NIH 3T3 fibroblasts and C2C12 myoblasts. These three types of cells were maintained in proliferation medium (Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 10% horse serum (HS), and 0.5% chicken embryo extract).

ELISA assay

A low serum-containing medium (DMEM supplemented with 1% FBS and 1% HS) was used to culture cells in the experiments of growth factor and cytokine expression. The media from the TGF-β1 (5 ng/ml, Sigma, St. Louis, MO) and PGE_2 (0, 100, 1,000, or 10,000 ng/ml) treatments experiment were collected and kept at −80°C pending enzyme-linked immunosorbent assay (ELISA). The assay was performed as suggested by the manufacturer's protocols (DE0100 PGE_2 ELISA kit, DE1150 $PGF_{2\alpha}$ ELISA kit, R & D Systems, Inc., Minneapolis, MN).

Animal model

The Animal Research and Care Committee at the authors' institution approved all experimental protocols for this study (Protocol 29/04). Twenty-four C57BL/6J mice (males, 6 weeks of age, Jackson Laboratories), 4 COX-2 knock-out mice (males, 6 weeks of age, Taconic farm), and their wild-type controls (males, 6 weeks of age, Taconic farm) were used for in vivo experiments. The GMs of the mice were injected with cardiotoxin (CTX, c3987, Sigma), a snake venom, to create a muscle injury. Briefly, the mice were anesthetized by intraperitoneal injection of 0.03 ml ketamine (100 mg/ml) and 0.02 ml xylazine (20 mg/ml). Ten microliters of diluted CTX (or 9μl CTX + 1μl TGF-β1) was injected in the middle mass of each GM. The mice were sacrificed at different time points after injection (1, 3, 5, or 14 days). The GMs were harvested from both legs for either flow cytometry or histological analysis. For the latter purpose, the GMs were fresh-frozen in 2-methylbutane pre-cooled by liquid nitrogen, and stored at −80°C pending cryosection.

Macrophage depletion

Clodronate liposomes, were prepared as described previously (Seiler et al., 1997; Tyner et al., 2005). The liposomes act as carriers for clodronate, which is toxic to phagocytic cells. Two days before muscle injury, 1 mg of clodronate liposomes (20 mg/ml) was injected intraperitoneally into C57/BL 6J mice to deplete macrophages. Mice injected with empty liposomes were used as controls. Flow cytometry using macrophage marker antibodies (F4/80 and CD-11b) was used to verify the extent of macrophage depletion.

Hematoxylin and eosin (H & E) staining

The cryosections were fixed in 1% glutaraldehyde for 1 min, and then immersed in hematoxylin for 30 sec. After washing with alcohol acid and ammonia water, they were immersed in eosin for 15 sec. After each step, sections were rinsed with distilled water. The sections were then dehydrated by treatment with alcohols of increasing concentrations (70%, 80%, 95%, and 100%). Following this, the sections were treated with xylene and covered with glass slips. Slides were analyzed on a bright field microscope (NIKON Eclipse E800, Nikon, Tokyo, Japan). Northern Eclipse software (Empix Imaging, Cheektowaga, NY) was used to measure the minor axis diameters of centronucleated regenerating myofibers (i.e., the smallest diameter of a myofiber across the central nucleus; 200× magnification; 200 random myofibers obtained from four samples/group).

Western blot

After washing with PBS, Laemmli sample buffer (BioRad, 161-0737, Hercules, CA) was applied to the surface of culture dishes to collect proteins from live cells. For muscle tissue samples, serial cryosections of 10 μm thickness were collected in Eppendorf tubes and treated with T-PER tissue protein extraction agent (78510, Pierce, Rockford, IL) and mixed with Laemmli sample buffer (BioRad, 161-0737). Protein samples were boiled for 5 min, separated on 10% SDS–polyacrylamide electrophoresis gels, and transferred to nitrocellulose membranes. Mouse anti-COX-2 (160112, Cayman, Ann Arbor, MI) and anti-TGF-β1 antibodies (555052, BD Biosciences Pharmingen, San Diego, CA) were applied as primary antibodies, and mouse anti-β-actin (Sigma; 1:8,000) was used for protein quantification. The horseradish peroxidase-conjugated secondary antibodies (Pierce) were diluted to 1:5,000 (v/v). Blots were developed by using SuperSignal West Pico Chemiluminescent substrate (Pierce), and positive bands were visualized on X-ray film. All results were analyzed by Northern Eclipse software (Empix Imaging).

Flow cytometry

The GMs from non-TGF-β1-treated (10 μl cardiotoxin injection) and TGF-β1-treated groups (9 μl cardiotoxin plus 1 μl of 5 ng/ml TGF-β1 injection) were surgically removed before injury, and at 1, 2, 3, and 5 days after injury for serial evaluation. Collagenase, dispase, and trypsin were used to digest the tissue matrix and

isolate the cells. Debris was removed via filtration with 70 μm filters.

Cells were treated with 10% mouse serum (Sigma) to block non-specific binding sites. Primary rat anti-CD-11b (conjugated with FITC, R & D Systems, Inc.) and rat anti-F4/80 (conjugated with APC, BD Biosciences, San Jose, CA) antibodies were used in combination to identify neutrophil and macrophage populations. We added 7-amino-actinomycin D (7-AAD; BD Biosciences Pharmingen, San Diego, CA) to exclude non-viable cells from the analysis. Samples were then analyzed via fluorescence activated cell sorting (FACS) Caliber flow cytometer (BD Biosciences) and CellQuest software (BD Biosciences).

Statistics

A Chi-squared analysis was used to analyze the percent differences between the number of macrophages identified by flow cytometry and western blotting results. Comparisons between two groups were made by the use of an unpaired Student's *t*-test. All other data were analyzed by one-way ANOVA statistical analysis. Post-hoc multiple comparison tests were performed to determine which means differed. A value of $P < 0.05$ was considered statistically significant. Error bars on figures represent the standard deviation.

Results

Macrophage depletion and muscle regeneration

Liposome clodronate was injected intraperitoneally 2 days prior to muscle injury to deplete the macrophage populations in mice. We used flow cytometry to quantify the number of infiltrating macrophages after injury. This macrophage infiltration peaked 2 days post-injury, and decreased quickly after 5 days. Liposome clodronate injection significantly reduced the number of infiltrating macrophages after muscle injury at all time points observed when compared to the empty liposome control group. At 1d, 2d, 3d, and 5d post-injury, liposome clodronate injection decreased macrophage infiltration by 73.2%, 80.2%, 77.4%, and 64.2%, respectively ($P < 0.05$, Fig. 1A). As shown on the flow cytometry graph, the macrophage population, which is shown in the S2 quadrant in the empty liposome control mice, was significantly reduced in the clodronate liposome-injected mice (Fig. 1B).

Clodronate liposome-treated mice exhibited reduced muscle regeneration at both 14 and 28 days after injury. The size of regenerating myofibers in liposome clodronate-treated mice was significantly smaller than those observed in the empty liposome control group ($P < 0.05$, Fig. 1C–E). However, at 7 days post-injury, there was no significant difference between these two groups.

Macrophages depletion decreases the expression of TGF-β1

Injured GMs were isolated from empty liposome control and clodronate liposome-injected mice. We examined the TGF-β1 expression level in injured muscle tissue by using Western blot analysis. Our results indicated that, compared to the empty liposome control group, TGF-β1 expression was significantly decreased 3 and 5 days after injury in the clodronate liposome-injected group, which had its macrophages depleted before the creation of muscle injury ($P < 0.05$, Fig. 2A,B). These results suggest that macrophages play an important role in inducing the expression of TGF-β1 during the inflammatory phase of skeletal muscle after injury.

TGF-β1 induces the production of COX-2 enzyme and prostaglandins

To examine the effect of TGF-β1 on the COX-2 pathway, we cultured MPCs and treated them with TGF-β1. We analyzed the COX-2 enzyme level from MPCs treated with TGF-β1 and the non-treated control cells for 4 days. Western blot analysis showed that COX-2 production was significantly higher in the MPCs treated by TGF-β1 compared to non-treated control cells ($P < 0.05$, Fig. 3A,B). We further tested the expression of PGE_2 from different muscle cells, including MPCs, NIH 3T3 fibroblasts, and C2C12 myoblasts, after they were treated with TGF-β1 for 4 days. We found that TGF-β1 treatment increased the expression of PGE_2 significantly in all cell types, when compared to non-treated control cells ($P < 0.05$, Fig. 3C).

To further validate the relationship between TGF-β1 and the COX-2 pathway, we used wild-type MPCs as a model system. In this experiment, NS-398 (a COX-2 specific inhibitor) was added to the cell culture to block the COX-2 enzyme activity. This additional treatment ablated the increased PGE_2 expression that was induced by adding TGF-β1 into the cell culture ($P < 0.05$, Fig. 3D). In addition, COX-$2^{-/-}$ cells had very low levels of PGE_2 expression, even if TGF-β1 was added. This finding suggests that TGF-β1 regulates the production of COX-2 pathway products, and that the enhanced expression of PGE_2 may be mediated by COX-2.

TGF-β1 increases the infiltration of macrophages through the COX-2 pathway

To examine the effect of TGF-β1 on macrophage infiltration, we created CTX injury (with or without TGF-β1 treatment) on the GMs of wild-type mice. We found that, after TGF-β1 treatment, the number of infiltrating macrophages was significantly increased compared with the CTX-only group at days 1, 2, and 3 after muscle injury ($P < 0.05$, Fig. 4A). The same experiment was conducted on COX-$2^{-/-}$ mice. Interestingly, we found that the number of infiltrating macrophages actually decreased with the addition of TGF-β1, instead of being increased 3 days after injury ($P < 0.05$, Fig. 4B). This further validates the suggestion that the effect of TGF-β1 on macrophage infiltration is mediated at least in part by the COX-2 pathway products.

PGE_2 decreased the expression of TGF-β1

To examine the influence of PGE_2 on fibrosis formation, we chose to examine the effect of PGE_2 treatment on the expression of TGF-β1, which is known for its fibrotic effect especially in skeletal muscle (Li et al., 2004). Three different muscle cell types, MPCs, NIH 3T3 fibroblasts, and C2C12 myoblasts, were tested for TGF-β1 expression following treatment with PGE_2 for 4 days. We found that PGE_2 treatment decreased the expression of TGF-β1 significantly in all cell types tested, when compared to non-treated control cells ($P < 0.05$, Fig. 5). This result suggests that PGE_2 may be able to decrease fibrosis formation after muscle injury by decreasing the expression of fibrotic growth factor TGF-β1.

Discussion

Inflammation is an important phase of skeletal muscle healing. Macrophages, TGF-β1, and the COX-2 pathway are the integral components of this phase. In order to further the understanding of muscle healing and improve the health care of muscle injury patients, we must investigate the importance of these components and elucidate their interaction in muscle healing regulation. In this study, we examined their roles and relationships during the inflammation phase and postulated a possible regulatory mechanism (Fig. 6).

Many researchers suggest that macrophages are important for muscle regeneration (Cantini et al., 2002; Camargo et al., 2003; Chazaud et al., 2003; Tidball, 2005). To create a "Loss of Macrophages Function" condition in skeletal muscle healing, especially in the inflammation phase, we used the clodronate liposome injection technique (Seiler et al., 1997; Tyner et al.,

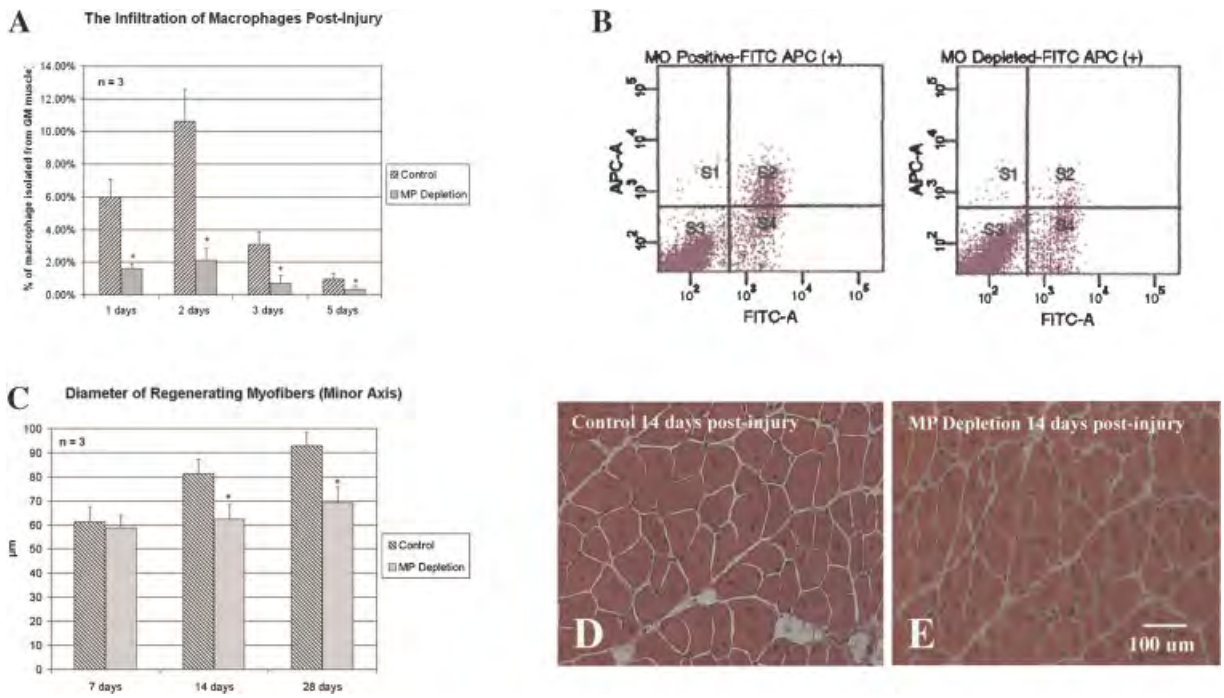

Fig. 1. Injection of clodronate liposome depleted macrophages in injured GM significantly after injury. At 1d, 2d, 3d, and 5d post-injury, liposome clodronate injection decreased macrophage infiltration by 73.2%, 80.2%, 77.4%, and 64.2%, respectively ($P < 0.05$, A). Flow cytometry results showed that the macrophage population, which was shown in the S2 quadrant in non-treated mice at 48 h after injury (B, left), was significantly reduced in clodronate liposome-treated mice (B, right). The X-axis represents the CD-11b cell surface marker; and the Y-axis represents the F4/80 cell surface marker. Compared with empty liposome-control group, clodronate liposome-injected mice exhibited significantly reduced sizes of regenerating myofibers 14 and 28 days post-injury ($P < 0.05$, C). Sample H & E stained GM sections from empty liposome and clodronate liposome-injected groups 14 days post-injury are shown in (D,E). The asterisks indicate a statistically significant difference ($P < 0.05$) between the marked groups and the control group. The error bars in the graph represent the standard deviation. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

2005) to deplete the macrophage populations in mice. With the injection of clodronate liposomes, most of the macrophages were depleted and only a few remaining cells infiltrated into muscle tissue during the inflammation phase. We found that, although regeneration of injured muscle did occur, the macrophage depletion group had significantly smaller myofibers than the non-treated group, which is indicative of a delay in muscle regeneration. This suggests that macrophages participate in the muscle healing process, and may play a beneficial role in the growth of regenerating myofibers.

One of the possible mechanisms by which macrophages contribute to the healing process is by inducing the proliferation and differentiation of satellite cells by secreting growth factors and cytokines (Cantini et al., 1994, 1995; Lescaudron et al., 1999; Merly et al., 1999; Chazaud et al., 2003). The factors secreted by macrophages exert their effects not only on specialized satellite cells during muscle regeneration, but have a broader mitotic activity on all myogenic cells (Cantini and Carraro, 1995). In this study, we found that macrophage-depleted mice had significantly lower TGF-β1 levels in their injured GM muscles compared to those from non-treated control mice. This finding suggests that macrophages play a role in modulating the level of these growth factors including TGF-β1 during the healing process of skeletal muscle. However, the exact role of macrophages and their interaction with other muscle cells warrants further investigation. Studies need to be performed to determine the cellular source of growth factors in the muscle inflammation process, and whether direct contact or humeral regulation is necessary to induce the expression of these growth factors.

Fibrosis is a complex biological process that is usually seen in severe muscle injury. Fibroblasts are activated to proliferate and produce abnormal amounts of extracellular matrix (ECM). Damaged skeletal muscle tissue is replaced by the deposition of overproduced ECM (i.e., fibrosis/scar) tissue, instead of regenerating myofibers. TGF-β1 is one of the most potent fibrotic stimuli (Li et al., 2004). It is an inducer of ECM protein synthesis and fibroblast proliferation (Kahari et al., 1991; Taipale et al., 1994), and it has been implicated in the fibrogenesis of various tissues (Border and Noble, 1994). However, the role of TGF-β1 in skeletal muscle healing is not limited to fibrosis. Previous studies have indicated that TGF-β1 may be an immunosuppressive molecule because its elimination or its downstream Smads signaling cascade disruption leads to severe inflammatory disease (Kulkarni and Karlsson, 1997; Yang et al., 1999; Nakao et al., 2000; Monteleone et al., 2004a). On the other hand, it has been suggested by some authors that TGF-β1 is able to increase PGE_2 expression in other tissues (Fawthrop et al., 1997; McAnulty et al., 1997; Keerthisingam et al., 2001; Han et al., 2004). Since it has been suggested that the COX-2 pathway products including PGE_2 and $PGF_{2\alpha}$ are important inflammatory mediators that induce regeneration in skeletal muscle healing (Bondesen et al., 2004; Shen et al., 2005), it is important to explore their relationship with TGF-β1. In our in vitro studies, we found that the addition of TGF-β1 significantly increased the production/expression of both COX-2 enzyme and PGE_2 in muscle cells. By blocking COX-2, TGF-β1's effect on PGE_2 expression was ablated. Furthermore, the effect of TGF-β1 was not observed in COX-$2^{-/-}$ cells. These results indicate that TGF-β1 is not only a fibrotic inducer,

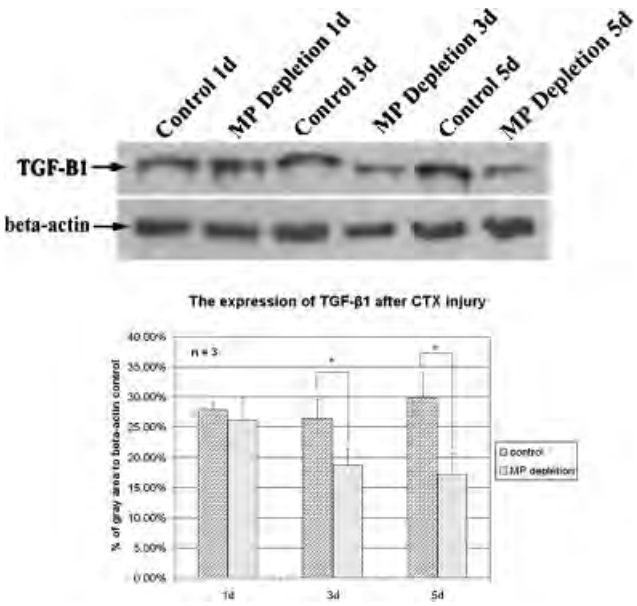

Fig. 2. The in vivo expression level of TGF-β1 was significantly lower in the clodronate liposome-injected mice compared to the empty liposome control mice 3 and 5 days after injury ($P < 0.05$). A representative western blot result is shown at the top. The asterisks indicate a significant difference ($P < 0.05$) between the compared groups. The error bars in the graph represent the standard deviation.

but is also an inflammatory modulator in muscle injury. In fact, TGF-β1 may enhance the inflammatory response by enhancing the COX-2 pathway, especially the production of PGE_2. This may represent an important finding because it indicates that TGF-β1 may both up- and downregulate inflammation through different pathways.

It has been shown that TGF-β1 interferes with the inflammation phase in various ways (Fiocchi, 2001; Warshamana et al., 2002; Monteleone et al., 2004a,b; Wang et al., 2005). Mostly, TGF-β1 was thought to inhibit inflammation because TGF-β1 is a negative regulator of NF-κB activation (Haller et al., 2003). Smad7 maintains high NF-κB activity during inflammation by blocking TGF-β1 signaling (Monteleone et al., 2004a,b; Wang et al., 2005). To elucidate whether TGF-β1 modulates inflammation by other means, macrophage infiltration for example, we injected TGF-β1 together with CTX. The TGF-β1 addition significantly increased the infiltration of macrophages into injured skeletal muscle. This suggests that TGF-β1 interferes with the inflammation phase of muscle healing by increasing the number of infiltrating macrophages. However, in COX-$2^{-/-}$ mice, the number of infiltrating macrophages was decreased with the addition of TGF-β1 to the cardiotoxin injection. This finding suggests that the COX-2 pathway mediates this TGF-β1-induced macrophage infiltration. As prostaglandins are known to cause localized vasodilation, it is reasonable to postulate that they may be at least partly responsible for the chemotaxis of macrophages. In future experiments, prostaglandin's chemotactic property as well as TGF-β1's regulation of NF-κB should be more deeply investigated. If it could be shown that TGF-β1 also has a negative impact on inflammation through the downregulation of NF-κB, it would be intriguing to discover which pathway is dominant in muscle injury, and determine why TGF-β1 would have contradictory effects. Based on the findings that macrophages increased the expression of TGF-β1, and TGF-β1 increased the infiltration of macrophages, which is mediated by the COX-2 pathway, they may form a positive feedback loop to further enhance the number of infiltrating macrophages in the injured muscle (Fig. 6).

A previous study of liver fibrosis showed that PGE_2 inhibited TGF-β1-mediated induction of collagen alpha I production in

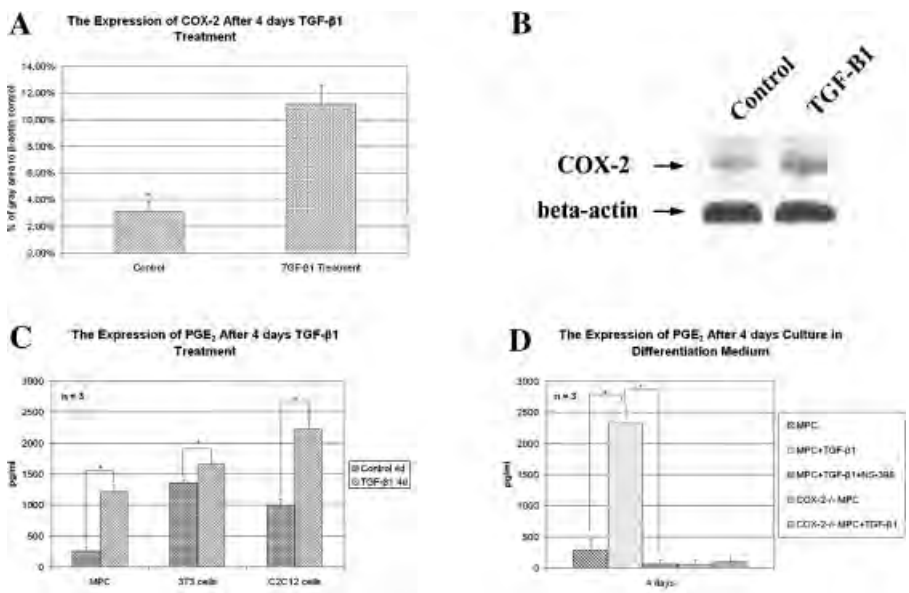

Fig. 3. MPCs that were treated with TGF-β1 for 4 days had significantly higher production of COX-2 enzyme than the non-treated control cells ($P < 0.05$, A). A representative Western blot result is shown in (B). Muscle cells that were treated by TGF-β1 for 4 days had significantly higher expression levels of PGE_2 compared with the non-treated control cells ($P < 0.05$, C). NS-398 can significantly reduce the increased PGE_2 expression that was induced by TGF-β1 ($P < 0.05$, D). COX-$2^{-/-}$ MPC cells, both treated and non-treated with TGF-β1, expressed PGE_2 at very low levels. The asterisks indicate a significant difference ($P < 0.05$) between the compared groups. The error bars in the graph represent the standard deviation.

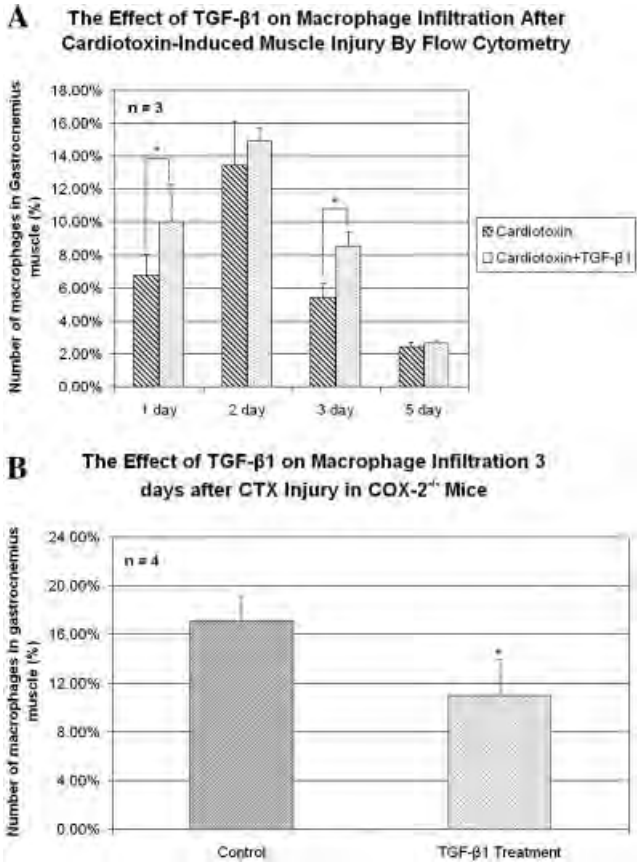

Fig. 4. Flow cytometry results showed that CTX injection can create injury in skeletal muscle and induce the infiltration of macrophages. The addition of TGF-β1 significantly increased the number of infiltrating macrophages at 1 and 3 days after CTX-induced muscle injury ($P < 0.05$, A). In contrast, CTX-injured COX-2$^{-/-}$ mice showed significantly decreased numbers of infiltrating macrophages 3 days post-injury ($P < 0.05$, B). The asterisks indicate a significant difference ($P < 0.05$) between the compared groups. The error bars in the graph represent the standard deviation.

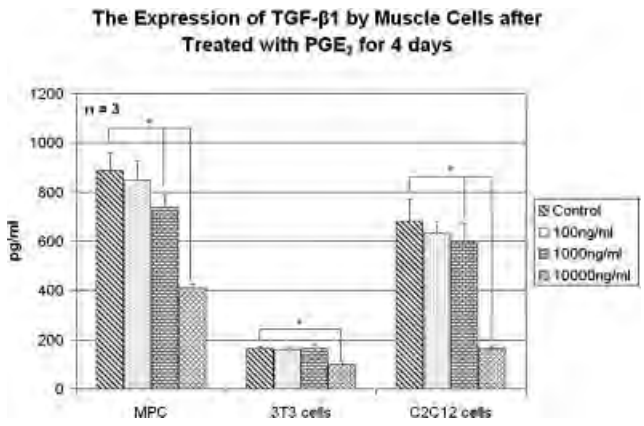

Fig. 5. At concentrations of 1,000 ng/ml and 10,000 ng/ml, PGE_2 decreased the expression of TGF-β1 significantly in both MPCs and C2C12 myoblasts. However, PGE_2 was able to decrease the expression of TGF-β1 in NIH 3T3 fibroblasts only at a concentration of 10,000 ng/ml ($P < 0.05$). The asterisks indicate a significant difference ($P < 0.05$) between the compared groups. The error bars in the graph represent the standard deviation.

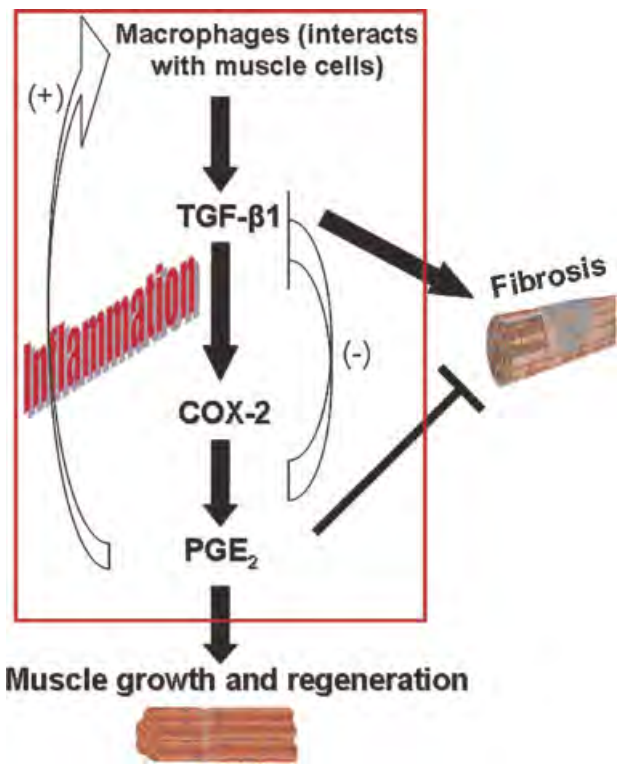

Fig. 6. The proposed mechanism of muscle inflammation is shown. Macrophages interact with muscle cells to increase the level of TGF-β1 in muscle inflammation. TGF-β1 increases the level of prostaglandins, which mediates muscle regeneration and the further infiltration of macrophages, forming a positive feedback loop. In turn, PGE_2 decreases the expression of TGF-β1 and forms a negative feedback loop to downregulate the fibrosis formation. Macrophages, muscle cells, TGF-β1, and COX-2 pathway form a complex network to regulate each other, and thus modulate the following regeneration and fibrosis formation during the healing process after injury. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

hepatic cells (Hui et al., 2004). This finding suggests that PGE_2 and TGF-β1 are able to regulate one another's levels by forming a negative feedback loop resulting in homeostasis of fibrosis formation. To test this phenomenon in skeletal muscle, we treated different muscle cell types with PGE_2 and analyzed their TGF-β1 expression. What we found was that PGE_2 treatment decreased the expression of TGF-β1 significantly in all muscle cell types, when compared to non-treated control cells. On the other hand, our previous study showed that by using NS-398 to block COX-2, and thus the expression of PGE_2, the expression of TGF-β1 was increased in injured muscle tissue (Shen et al., 2005). These results indicate that the TGF-β1 level was probably regulated by PGE_2. The application of NS-398 probably inhibited PGE_2 expression and led to a high level of TGF-β1, and subsequent fibrosis formation in injured muscle. Our findings are consistent with the results from a previous liver fibrosis study (Hui et al., 2004), and further suggests the existence of a negative feedback loop between TGF-β1 and PGE_2 in skeletal muscle (Fig. 6).

PGE_2 is also a potent inhibitor of fibroblast proliferation (Goldstein and Polgar, 1982; Durant et al., 1989) and collagen

synthesis (Goldstein and Polgar, 1982; Saltzman et al., 1982). This suggests that PGE_2 may play an important role in minimizing ECM production. This is especially important in an environment favoring fibrosis formation, such as is found within the inflammation of damaged liver. Interestingly, our preliminary data showed that different concentrations of PGE_2 may affect the proliferation of muscle cells differently (data not shown). Thus, the next step in our study would be to assess the level of PGE_2 in injured skeletal muscle, which should give further insight into the effect of PGE_2 on muscle cell proliferation. It is also important to investigate the role of other prostaglandins, such as $PGF_{2\alpha}$, PGE_2, and 15-PG dehydrogenase in the future, because other prostaglandins may also play a role in the muscle healing process, and 15-PG dehydrogenase may have a significant effect on prostaglandin levels.

In summary, our study demonstrates that the COX-2 pathway, macrophages, and TGF-β1 are important components of the inflammation phase of injured skeletal muscle. Inflammation affects the overall healing of skeletal muscle through these cellular and molecular components. In addition, we found that these components may modulate the production of each other and form a complex co-regulatory mechanism. As we begin to comprehend the fact that simply blocking inflammation by using NSAIDs has a potential downside on muscle healing, more studies are warranted to improve the quality of healthcare in patients afflicted with skeletal muscle injuries.

Acknowledgments

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Blocking Myostatin by AAV2-Delivered Myostatin Propeptide Improves Muscle Cell Transplantation

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INTRODUCTION

Myostatin (MSTN) is a potent negative regulator of muscle growth [1] and we have found that like TGF-β1, MSTN also contributes to the formation of fibrosis in injured skeletal muscle [2]. JacqueTremblay's group has successfully blocked MSTN signaling in *mdx* mice by generating 2 types of transgenic *mdx* mice. The 1st carries a dominant negative form of the MSTN receptor (dnActRIIB) and the 2nd over-expresses follistatin (FSTN), which is an inhibitor of MSTN. Normal myoblasts transplanted into these transgenic *mdx* mice outperformed myoblasts transplanted into nontransgenic *mdx* mice [3, 4]; nevertheless, dnActRIIB and FLST do not specifically inhibit MSTN but also interfere with other growth factors such as activins. In the current study we utilized an adeno-associated viral vector carrying the MSTN propeptide gene (AAV-MPRO) to specifically inhibit the action of MSTN. MPRO directly binds the MSTN molecule and has been shown in previous studies. We have proved that blocking MSTN with MPRO both increase muscle regeneration and reduce the formation of fibrosis at the site of injury 4 weeks following injury (unpublished data). In the current study we injected AAV-MPRO/GPF into the skeletal muscles of *mdx*/SCID mice 4 weeks prior to muscle progenitor cell transplantation to investigate whether blocking MSTN signaling in host muscle by MPRO can elevate the regeneration capacity of donor muscle cells in dystrophic muscle. The success of cell therapy for treating muscle injuries and diseases has been limited by the poor survival and function of the donor cells after transplantation; therefore, improving the microenvironment in the host dystrophic muscle prior to cell transplantation is an alternative approach to enhance the efficiency of cell transplantation.

METHODS

Animal model: All experiments in this study were approved by the Children's Hospital of Pittsburgh IACUC. AAV2-MPRO ($1x10^{11}$ v.g.) in 50 µl of PBS was injected into the gastrocnemius muscles (GMs) of 3 *mdx*/SCID mice (4 weeks of age); the same amount of AAV-GFP was injected into the GMs of mdx/SCID littermates as controls. Four weeks after injection of AAV, 300,000 muscle progenitor cells (MPCs) were injected into each of the GMs of AAV treated *mdx*/SCID mice (n = 6), and the mice were sacrificed 2 weeks following the cell transplantation. Masson's Trichrome staining was performed to identify fibrous scar tissue in the mice. Northern Eclipse software (Empix Imaging, Inc.) was used to measure the diameters of regenerating myofibers within the injection site and also measure the areas of fibrous scar tissue. Dystrophin staining was performed and the dystrophin-positive myofibers were then counted to assess the efficiency of cell transplantation in the skeletal muscle of the *mdx*/SCID mice. Student's *t* test was used to determine significance ($P<0.05$).

Immunohistochemistry: Muscles were snap frozen in liquid nitrogen, cyrosectioned and the tissue sections were then fixed in 5 % formalin for 5 minutes, followed by 3 washes with PBS. The sections were blocked with 10% donkey serum for 1 hour, and incubated overnight at 4°C in either a goat MPRO antibody (2.5 ug/ml, RnD system) or a rabbit dystrophin primary antibody (1:500, Abcam) that was diluted in 5% donkey serum. The following day, the sections were washed with PBS and incubated with the corresponding secondary antibodies, rabbit anti-Goat or donkey anti-rabbit IgG conjugated with Alexa594.

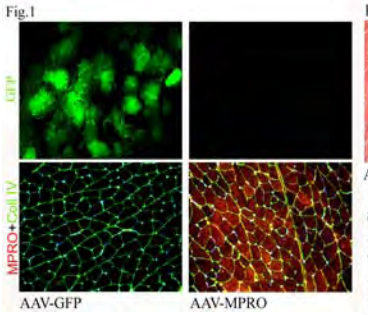
RESULTS

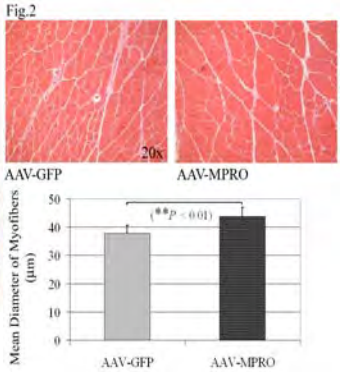
Gene expression in muscle: Expressions of GFP (green) and MPRO (red) in AAV-GFP/MPRO transduced muscles indicates that genes delivered by AAV were stably expressed in skeletal muscle (Fig. 1).

Accelerated muscle regeneration in MPRO over-expressing mdx/SCID mice: Six weeks after AAV2-MPRO transduction, a significant increase in muscle weight was observed compared to the GFP transduced control (data not shown). Moreover, the mean diameter of the muscle fibers in the AAV-MPRO transduced muscles was significantly larger than that of AAV-GFP transduced muscles (Fig. 2).

Reduced fibrosis: Masson's trichrome stain showed a reduction of fibrous scar tissue (blue) in the AAV2-MPRO transduced GMs as compared to the AAV-GFP control muscles (Fig. 3).

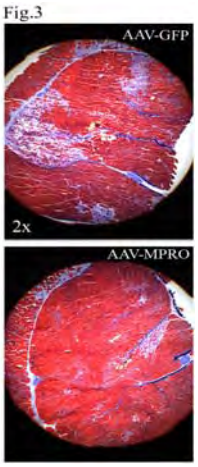
Improved cell transplantation in the GMs of AAV-MPRO transduced mdx/SCID mice: Fig.4 shows the representative images of dystrophin-positive muscle fibers in the muscles of *mdx*/SCID mice 2 weeks after cell transplantation. Normal MPCs injected into AAV-MPRO transduced dystrophic muscle surpassed the regeneration capacity of cells injected into AAV-GFP transduced control muscle. This was determined by counting the total number of dystrophin positive myofibers (Fig. 4)

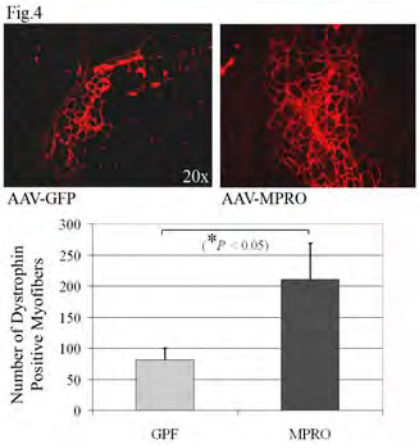




DISCUSSION

Our results revealed stable expression of AAV-mediated GFP and MPRO in transduced skeletal muscle. MPRO ameliorated the dystrophic pathology of *mdx* mice by promoting muscle regeneration and reducing collagen deposition. Inactivation of MSTN in dystrophic host muscle by MPRO significantly improved the success of MPC transplantation as compared to the AAV-GFP transduced control, evidenced by significantly more dystrophin-positive myofibers in the former. Two mechanisms may account for this improved cell transplantation. First, MPRO counteracts the inhibitory effect that MSTN has on donor cell regeneration. Deregulating the suppression of MSTN on muscle cells by MPRO transduction may augment donor cell proliferation, differentiation, and hence increase dystrophin-positive myofiber formation. Second, the improved cell transplantation in *mdx* mice by MPRO may be partially accredited to the fact that MPRO inhibits MSTN, thereby reducing fibrosis formation in dystrophic muscle. The ameliorated milieu in host dystrophic muscle may favor donor cell survival and engraftment. Taken together, the combination of gene therapy and cell therapy has been shown to be a potential effective and novel approach for treating injured and diseased muscle.





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Angiotensin II Receptor Blocker Ameliorates Skeletal Muscle Healing in a Dose Dependent Manner

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INTRODUCTION:

Muscle injuries are very common musculoskeletal problems encountered in sports medicine. Although these injuries are capable of healing, complete functional recovery is hindered by the formation of dense scar tissue triggered by TGF-β1 [1]. We have previously reported that several agents such as decorin and suramin can inhibit fibrosis and improve regeneration in injured skeletal muscle. The safety of these agents, however, remains unknowns to use those agents for treating muscle injury. By contrast, Losartan (LOS), one of the Angiotensin II Receptor Blockers (ARBs)—is a FDA approved antihypertensive medication and has been shown to also be antifibrotic in a variety of tissues, including skeletal muscle [2]. This ARB has a well-tolerated side-effect profile, can also block TGF-β1 to attenuate the development of pathological fibrosis. In this study, we investigated optimum dose of LOS in treating injured muscle to help the translation of this research from bench to bedside.

METHODS:

Cell proliferation assay: C2C12 myoblasts and fibroblasts obtained from murine skeletal muscle by using the previously published preplating technique [3], were cultured in 96 well plates (n=5) with DMEM containing 10% FBS,1% penicillin/streptomycin (P/S), with addition of different concentration of either Angiotensin II (ANG) (10^{-12} to 10^{-4} M) or LOS (10^{-12} to 10^{-4} M). CellTiter Cell Proliferation Assay kit (Promega, Madison, WI) was used to measure cell proliferation at 24, 48, and 74 hour after incubation.

C2C12 cell differentiation assay: C2C12 myoblasts were cultured in 24 well plates (n=4) with differentiation medium (DMEM supplemented 2% horse serum and 1% P/S) containing different concentration of ANG (10^{-12} to 10^{-4} M) or LOS (10^{-12} to 10^{-4} M). Three days after incubation immunocytochemistry of myosin heavy chain was performed and the fusion index (a ratio of the number of nuclei in myotubes/total nuclei) was calculated to assess the effects of ANG and LOS on differentiation capacity of C2C12 myoblast.

Animal model: The muscle contusion model was developed in tibialis anterior (TA) muscle of C57BL/6 wild-type mice. Different concentrations of LOS (12.5, 125, 1250, or 4000mg) in 1Liter of tap water were administered beginning immediately after injury until endpoint while the control group drink tap water (six mice in each group). These doses were calculated based on the average fluid intake of mice as 3, 30, 300, and 1000mg/kg/day respectively. Animals were sacrificed and TA muscles were harvested 4 weeks after injury. Muscle samples were then cryosectioned and histological stained (hematoxlin and eosin stain (H&E) and Masson's Trichrome stain). The numbers of centronucleated regenerating myofibers were counted to evaluate the regeneration.Northern Eclipse software (Empix Image, Inc.) was used to quantitate the total cross-sectional area of fibrosis. Statistical analysis was performed with ANOVA and Scheffe's F test as post hoc test. Statistical significance was defined as $p < 0.05$.

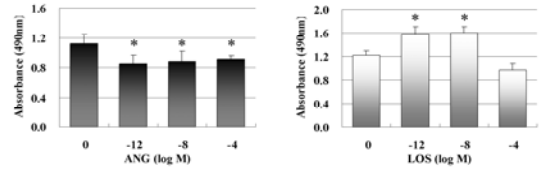
RESULTS SECTION:

LOS enhanceed C2C12 myoblast proliferation: Exposure to 10^{-12} to 10^{-4} M of ANG attenuated C2C12 proliferation after 72hr incubation. Whereas, 10^{-12} to 10^{-8} M of LOS stimulated C2C12 proliferation at the same time point. However, a high concentration of LOS (10^{-4} M) failed to increase cell growth (Fig. 1). Surprisingly, neither ANG nor LOS had any effect on fibroblast proliferation (not shown).

LOS stimulated myoblast differentiation: C2C12 differentiation was inhibited by 10^{-12}M of ANG, however, the effect attenuated at higher concentrations (Fig.2). Likewise, 10^{-12} M of LOS enhanced C2C12 differentiation and the stimulation was dropping while concentrations of LOS increased. It is noteworthy that 10^{-4} M of LOS significantly suppressed differentiation (Fig. 2).

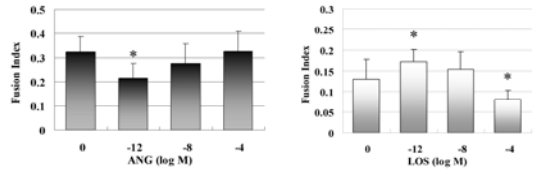
LOS enhanced muscle regeneration and decreased fibrosis: We could observe significant increases in number of centronucleated myofibers in the LOS-treated animals when compared with control animals (Fig. 3). The group treated with 300 mg/kg/day of LOS showed the most effective regeneration among groups. The best regeneration correlated with the group that displayed a significant reduction in fibrosis. However, these beneficial effects decreased when the dose of LOS was increased to 1000 mg/kg/day (Fig. 4).

Figure. 1



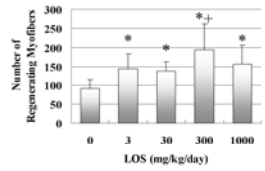
*: $p < 0.05$ vs control

Figure. 2



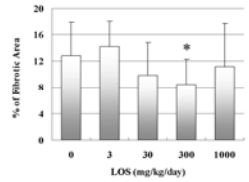
*: $p < 0.05$, vs control

Figure. 3



*: $p < 0.05$, vs control
+: $p < 0.05$, vs other trreated group

Figure. 4



*: $p < 0.05$, vs control

DISCUSSION:

The biphasic effect of LOS on C2C12 myoblasts *in vitro*, stimulating at low dose while decreasing at high dose, suggested that there is an optimal dose of LOS. Consequently we found that LOS improves skeletal muscle regeneration at 4 weeks after contusion injury, except that these effects were reduce/eliminated in 1000 mg/kg/day group. The best effective dose was 300 mg/kg/day. Overall, these effects of LOS were more pronounced for regeneration than for fibrosis. These *in vivo* results are consistent with our *in vitro* results that LOS was able to exert effects on C2C12 whereas fibroblasts were not affected by either ANG or LOS. Regeneration and fibrosis are two competitive processes after muscle injury; therefore, decreasing fibrosis observed in LOS-treated group is can be the result of the increase regeneration. In other words, LOS might indirectly reduced fibrosis by directly stimulating regeneration. Although there were not statistical differences in fibrosis in lower dose LOS groups (3 and 30 mg/kg/day) as compared to control, these effects of LOS on both regeneration and fibrosis showed similar dose dependent trend. However, *in vivo* results above were obtained only from single time point after injury. Since there are time lags between peaks of myofiber regeneration and fibrosis after injury, further investigations are required to examine the effect of LOS on regeneration and fibrosis at their individual peak time, which will facilitate clinical application of ARBs in improving skeletal muscle healing.

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Blocking Myostatin Improves Muscle Healing Via Enhancement of Angiogenesis

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INTRODUCTION

Myostatin (MSTN), a member of the TGF-β superfamily, is a powerful inhibitor of skeletal muscle growth [1], and has also been implicated in the formation of fibrosis in injured skeletal muscle [2]. An increase of muscle regeneration and a decrease of fibrosis is observed in the injured muscle of MSTN knockout (MSTN-/-) mice when compared to that of injured wild-type (WT) mice [2]. We have also observed similar results when we block MSTN utilizing adeno-associated viral vector to deliver MSTN propeptide (AAV-MPRO) (unpublished data), a natural inhibitor of MSTN [3]. In this study, we investigated whether AAV-MPRO could stably express in vivo and benefit the healing of injured skeletal muscle up to one year after muscle injury. We further examined mechanisms by which the inhibition of MSTN improved skeletal muscle healing after laceration injury. A positive correlation has been reported between angiogenesis and skeletal muscle healing [4]; therefore, we hypothesized that injured MSTN-/- muscles and injured muscles transduced with AAV-MPRO would show more angiogenesis than their respective controls.

METHODS

Animal experiments: We injected AAV-MPRO ($1x10^{11}$ v.g.) in 50 µl of PBS into gastrocnemius muscles (GMs) of C57BL/6 WT mice (6-8 weeks old). AAV-GFP was injected into GMs of WT littermates as a control. One month after AAV vector transduction, the GMs of the mice were lacerated. The GMs of 10 mice were harvested at 4 weeks post-injury (n = 5). The remaining mice were sacrifice at 1 year post-injury (n = 4). HE staining was performed to monitor muscle regeneration in the injured muscles. All samples were stored in -80°C.

Immunohistochemistry: The muscles were cryosectioned and fixed in formalin. The sections were then incubated with 10% HS for 1 hour after which a rat CD31 (endothelial marker) primary antibody (1:150) was applied along with a mouse anti-fast myosin heavy chain (MyHC) antibody or sheep anti-MPRO antibody, which were incubated for 1 hour at RT. The sections were then washed three times with PBS and incubated with the secondary antibodies rabbit anti-rat conjugated with 555 (red) and anti-mouse conjugated with 488 (green) or anti-sheep-555 (red) for 1h. Nuclei were counter stained with DAPI (blue). We used image J software to measure the number of CD31-positive capillaries to evaluate angiogenesis within the injury site. Student's *t* test was used to determine significance ($P<0.05$).

RESULTS

Expression of GFP and MPRO in skeletal muscle: One year after AAV transduction, we still detected strong expressions of GFP and MPRO in myofibers of AAV-GFP/MPRO transduced muscles (Fig. 1). MPRO-positive myofibers (arrowheads) indicates constitutive expression of MPRO in skeletal muscle transduced with AAV-MPRO.

Improved skeletal muscle healing by MPRO: We observed significantly larger regenerating myofibers in AAV-MPRO transduced injured muscle than in controls, 1 year post-injury (Fig. 2A, C). Correspondingly, distribution of diameter of regenerating myofibers revealed that AAV-MPRO transduced injured muscle contained a higher percentage of larger diameter regenerating myofibers. For instance, 30% of the diameters of regenerating myofibers in control muscle were larger than 50 µm when compared to 61 % of that in AAV-MPRO transduced muscle (Fig. 1B). Blocking MSTN with MPRO led to a significant increase in the weight of the GMs (Fig. 2D).

Increased angiogenesis in AAV-MPRO-transduced mice after GM laceration: Fig.3 shows representative images from injured AAV-MPRO transduced muscles and controls. Significantly more CD31-positive capillaries were observed in AAV-MPRO transduced injured muscle than in controls at 4 weeks post-injury (Fig.3).

DISCUSSION

Our unpublished data show that angiogenesis occurs earlier in the MSTN-/- muscle than in normal muscles after laceration injury. At 4 weeks after injury, MSTN-/- muscle contained significantly more CD31-positive capillaries than the controls. The increased angiogenesis appears to partially account for improved muscle healing in injured MSTN-/- mice. Nevertheless, it is noteworthy that the irreversibility of genetic transfer may result in compensatory upregulation, developmental defects, etc. We therefore used AAV to deliver MPRO cDNA into the GMs of WT mice in order to restrict MSTN propeptide over-expression in the certain skeletal muscles of adult mice. With this model, we injected AAV-MPRO particles into skeletal muscle prior to creating a laceration injury in the mice. Our results suggest that AAV-MPRO could be stably expressed in vivo and could improve the healing of the injured muscle, long term. We found that MPRO gene transfer improved skeletal muscle healing with increased muscle regeneration. Muscle transduced with MPRO also showed a greater amount of angiogenesis than untransduced controls which coincided with the increased regeneration and decreased fibrosis 4 weeks after injury (data not shown). Taken together, this data suggests a negative correlation between MSTN and angiogenesis in injured skeletal muscle; however, the clear relationship between MSTN and angiogenesis warrants further investigation.

Fig.1

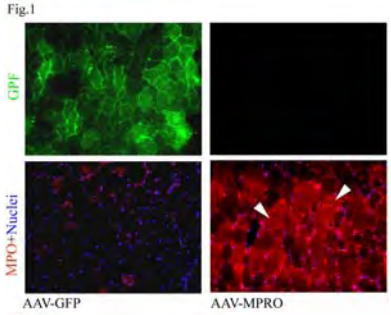


Fig.2

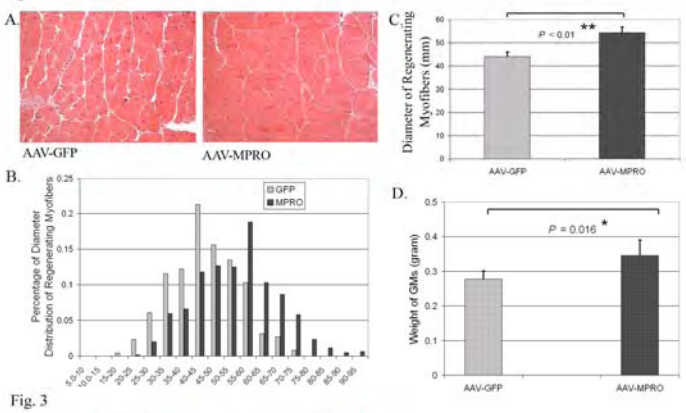
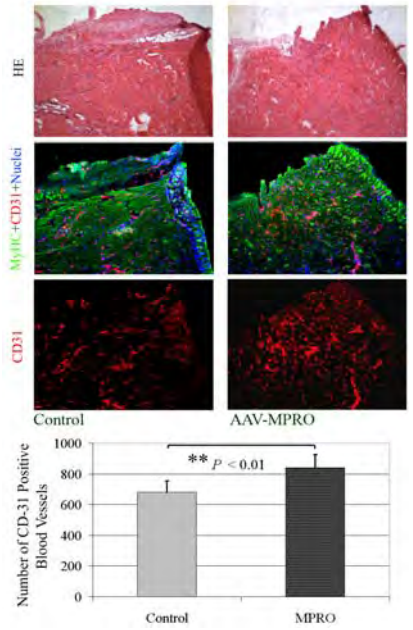


Fig. 3



ACKNOWLEDGMENTS

This work was supported by funding from the Henry J. Mankin Endowed Chair for Orthopaedic Research at the University of Pittsburgh, the William F. and Jean W. Donaldson Chair at Children's Hospital of Pittsburgh, the Hirtzel Foundation, and the National Institutes of Health (R01 AR47973 awarded to J.H.).

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Angiotensin Receptor Blocker Improves Skeletal Muscle Function Recovery in a Dose Dependent Manner

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INTRODUCTION:
Muscle injuries are very common musculoskeletal problem encountered in sports medicine. Although these injuries are capable of healing, complete functional recovery is hindered by the formation of dense scar tissue triggered by TGF-β1 [1]. We have previously reported that Losartan (LOS), one of the Angiotensin II Receptor Blockers (ARBs) FDA approved for antihypertensive treatment, has been shown to improve muscle healing through antifibrotic action [2]. We also demonstrated that specific doses of LOS (30 mg/kg/day and higher) improved muscle regeneration and attenuated the development of pathological fibrosis when were administrated immediately after injury [3]. In this study, we investigated whether LOS can improve muscle strength recovery after contusion injury, and also attempted to understand the mechanism of LOS action by analyzing gene expression of myostatin and follistatin, which are considered important regulators of skeletal muscle growth.

METHODS:

Animal model: The muscle contusion was developed in tibialis anterior (TA) muscle of C57BL/6 wild-type mice. Different concentrations of LOS (12.5, 125, 1250, or 4000mg dissolved in 1 liter of tap water) were administered to animals immediately after injury (n=6 in each group). These doses were calculated based on the average fluid intake of mice as 3, 10, 30, 300 mg/kg/day respectively. The control-injured mice received tap water. After 4 weeks animals were sacrificed to evaluate the healing.

Physiological testing: Under general anesthesia, the TA distal tendon was exposed and tied securely to a lever arm of transducer. Peroneal nerve was electrically stimulated and the specific peak twitch and tetanic force developed by TA muscle was monitored. All data were digitally recorded and stored until evaluation.

Quantitative Real-time PCR: Total RNA was extracted from muscle tissues using Nucleospin RNA kit (Clontech). cDNA was synthesized with SuperScriptTM II reverse transcriptase (Invitrogen), according to manufacturer's instructions. cDNAs and primers were added to SYBR Green PCR master mix (Applied Biosystem) according to manufacturer's instructions. The quantitative analysis for follistatin and myostatin gene expression was performed. All data were normalized to cyclophilin which was used as the internal control.

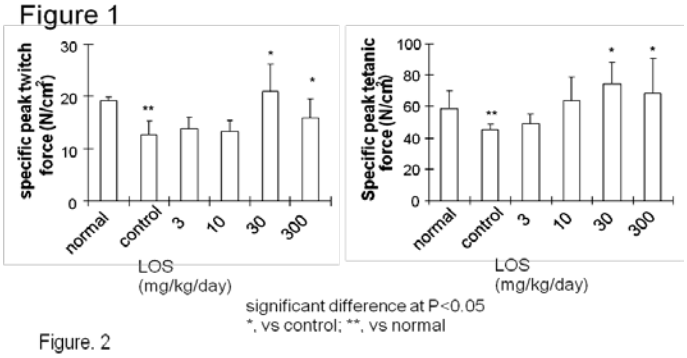
Statistical analysis: The results were expressed as the mean ± SD. To determine minimum effective dose of LOS in physiological testing, we analyzed the results using Williams' multiple comparison. The differences of mean specific twitch and tetanic force between control muscle (injured/non-treated) and normal muscle (non-injured) were analyzed with student t-test. Differences of gene expression between samples were assessed by the ANOVA and Tukey's test as post hoc test. Statistical difference was defined as $p < 0.05$.

RESULTS SECTION:

Improvement of Muscle Strength: LOS improved TA muscle force recovery after contusion injury. Specific peak twitch force and tetanic force was elevated in mice receiving high dose of LOS (30 and 300 mg/kg/day) in comparison to the animals receiving low dose of LOS (3 and 10 mg/kg/day) (Figure 1).

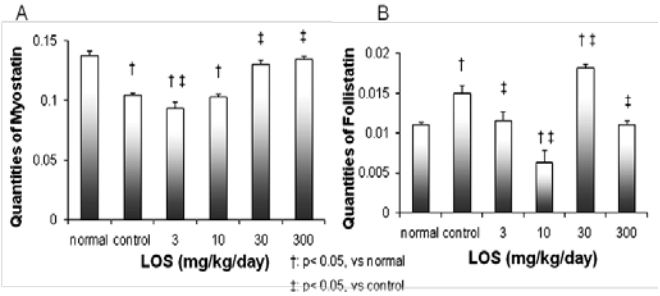
Myostatin gene expression: Expression of myostatin in the injured TA muscle in the control-injury and low dose of LOS treatment groups was lower than in the normal TA muscle, while there was no difference between normal and high dose of LOS treatment groups. Expression of follistatin in the control group was higher than in normal muscle and low dose of LOS treatment groups. The highest expression of follistatin was observed in the 30 mg/kg/day of LOS treatment group and it was significantly higher compared to the control group (Figure 2).

Figure 1



significant difference at P<0.05
*, vs control; **, vs normal

Figure. 2



DISCUSSION:
Functional recovery is the most important factor in the skeletal muscle healing after injury. Here we demonstrate that LOS administration immediately after injury improves recovery of skeletal muscle strength. These results also support our previous histological findings [3]. We believe that the mechanism of muscle regeneration after injury might be related to the expression of follistatin, positive regulator of skeletal muscle growth. We noticed over expression of follistatin in the 30 mg/kg/day treatment group compared to the normal and control-injury groups. These findings correlate with the results of physiological testing. It is unclear why the 30 mg/kg/day LOS display on increase expression of myostatin, negative regulator of skeletal muscle growth. Our results suggest that continuous administration of the high dose of LOS, in particularly 30 mg/kg/day, immediately after skeletal muscle injury could accelerate skeletal muscle functional recovery. We aimed to evaluate only single time point of LOS administration immediately after injury. Further studies are required to determine biological effect of LOS and facilitate clinical application of ARBs for improvement of skeletal muscle healing.

ACKNOWLEDGEMENTS:
The authors are grateful for technical assistance from Jessica Tebbets, Joseph Feduska, Michelle Witt, Richardo Ferrari, Avidas Usas and Burhan Gharaibeh. Funding support was provided by a grant from the Department of Defense (W81XWH-06-1-0406).

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Angiotensin II Receptor Blocker Ameliorates Skeletal Muscle Healing

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INTRODUCTION:
Muscle injuries are very common musculoskeletal problem encountered in sports medicine. Although these injuries are capable of healing, complete functional recovery is hindered by the formation of dense scar tissue triggered by TGF-β1 [1]. Losartan (LOS) is a FDA approved antihypertensive medication and has a well-tolerated side-effect profile. Our previous study revealed that 30 mg/kg/day of LOS treatment was effective in promoting muscle healing and inducing antifibrotic effect in a murine model of skeletal muscle after injury [2,3]. However, the effective dose (30 mg/kg/day) which was administrated immediately after muscle injury is higher compared to the dose used in human (10 mg/kg/day). In this study we investigated the effect on muscle healing in a murine animal model using human dose of LOS (10 mg/kg/day) administered at different time after injury.

METHODS:

Animal model: The muscle contusion model was developed in tibialis anterior (TA) muscle of C57BL/6J wild-type mice. The administration of 125 mg of LOS dissolved in 1 liter of tap water was started on day 0, 3, 7 and 14 after injury and continued for 4 weeks. Tap water without LOS was given to the animals in control-injury group (6 mice in each group). The LOS dose taken was calculated based on the average fluid intake of mice [3]. Four weeks after injury, we performed physiological testing and harvested the TA muscle for histology.

Physiological testing: Under general anesthesia, the TA distal tendon was exposed and tied securely to a lever arm of transducer. Peroneal nerve was electrically stimulated and the specific peak twitch and tetanic force developed by TA muscle was monitored. All data were digitally recorded and stored until evaluation.

Histology: TA muscles harvested 4 weeks after injury were cryosectioned and stained for hematoxlin and eosin (H&E) and Masson's Trichrome stain. The number of centronucleated regenerating myofibers was counted to evaluate muscle regeneration. Northern Eclipse software (Empix Image, Inc.) was used to analyse the total cross-sectional area of muscle fibrosis.

Quantitative Real-time PCR: Total RNA was extracted from muscle tissues using Nucleospin RNA kit (Clontech). cDNA was synthesized with SuperScriptTM II reverse transcriptase (Invitrogen), according to manufacturer's instructions. cDNAs and primers were added to SYBR Green PCR master mix (Applied Biosystem) according to manufacturer's instructions. The quantitative analysis for Follistatin (FSTN) and Myostatin (MSTN) gene expression was performed. All data were normalized to cyclophilin which was used as the internal control.

Statistical analysis: Differences between samples were assessed by the ANOVA and Scheffe's F test as post hoc test. Statistical significance was defined as $p < 0.05$.

RESULTS:

LOS enhanced muscle regeneration and reduced fibrosis: We observed significant increases in the number of centronucleated myofibers in the day 3 treatment group when compared with other treatment groups (Fig. 1). The highest effect on muscle regeneration coincided with significant decrease of fibrosis in the day 3 treatment group (Fig. 2).

LOS enhanced muscle force: LOS improved muscle strength recovery after contusion injury. Specific peak twitch force and peak tetanic force was significantly greater in mice treated with LOS beginning at day 3 after injury (Fig. 3).

LOS enhanced expression of Folistatin and Myostatin: Expression of FSTN detected by RT-PCR in the day 3 and day 14 LOS treatment groups was greater than in the normal non-injured or LOS treated at day 0 and day 7 groups (Fig. 4A). Expression of MSTN in the day 3, day 7 and day 14 LOS treatment groups was lower than in the normal, control and day 0 LOS treatment groups (Fig. 4B). The highest expression of FSTN coincided with the lowest expression of MSTN in the day 3 LOS treatment group.

Figure 1

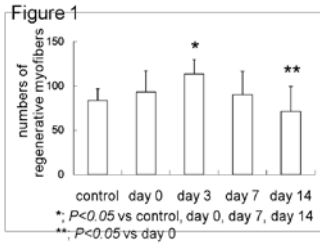


Figure 2

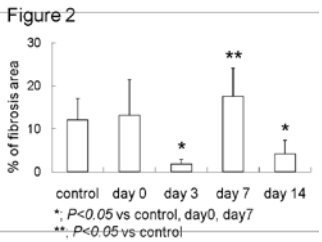


Figure 3

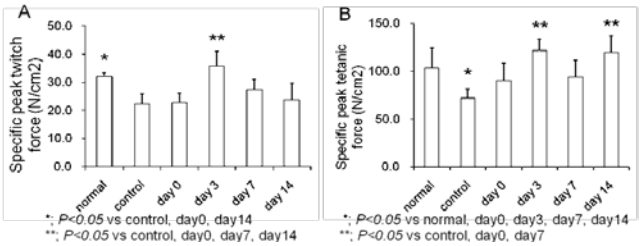
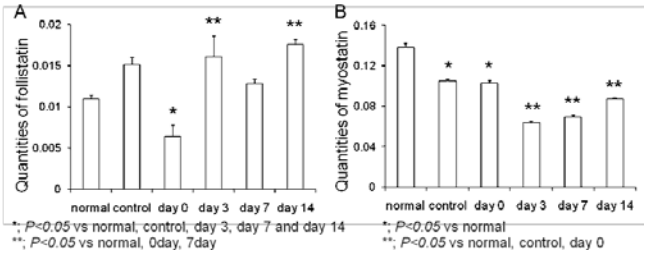


Figure 4



DISCUSSION:
Our study revealed that the most effective timing for administration of human dose of LOS (10 mg/kg/day) was 3 days after muscle injury. We observed increased number of centronucleated myofibers and decreased area of fibrosis when LOS was administered at day 3 after injury. The functional recovery after skeletal muscle injury is the most important factor for clinical translation of this therapy. We demonstrate that enhancement of muscle strength in day 3 LOS treatment group correlates with the improvement of muscle regeneration and the reduction of fibrosis. We have previously reported that muscle regeneration and fibrosis formation are two concomitant processes after muscle injury, and the effect of LOS was more prominent on muscle regeneration than on fibrosis [3]. Our study supports this finding. In addition, it may suggest that administration of LOS effectively leads to enhanced muscle regeneration after muscle injury via down regulation of endogenous MSTN, negative regulator of skeletal muscle growth. A decrease in FSTN expression was also observed in the day 3 LOS treatment group which may be related to the decrease in MSTN expression. In summary, we indicate that 10 mg/kg/day (human safety dose) of LOS treatment initiated at 3 days after contusion injury can enhance structural and functional healing in mouse skeletal muscle.

ACKNOWLEDGEMENTS:
The authors are grateful for technical assistance from Jessica Tebbets, Joseph Feduska, Michelle Witt, Richardo Ferrari, Aiping Lu, Avidas Usas and Burhan Gharaibeh. Funding support was provided by a grant from the Department of Defense (W81XWH-06-1-0406).

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Appendix 15

Improving Recovery Following Recurrent Hamstring Injury Using an Angiotensin II Receptor Blocker: Two Case Studies

Journal:	*Orthopaedic Research Society*
Abstract ID:	Draft
Presentation Type:	Either Poster or Podium
Categories for Abstract Review:	Muscle/Nerve - Muscle, Trauma - Clinical trauma research, Gait and Kinematics - Gait, Kinematics, Kinesiology
Keywords for Transactions on DVD-ROM:	Clinical Outcomes Research, Muscle, Gait, Kinematics, Kinesiology

Improving Recovery Following Recurrent Hamstring Injury Using an Angiotensin II Receptor Blocker: Two Case Studies

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INTRODUCTION

Hamstring muscle injuries are common in young competitive athletes. A recent study on National Football League training camp injuries reports hamstring injuries were the most common among muscle strain as well as the most severe. Recurrence is common, secondary to the pressure to return to play prior to complete healing of the injury, with a higher re-injury rate than any other type of injuries. Although muscles can undergo regeneration after injury, the healing process is slow and often culminates in incomplete functional recovery and formation of fibrosis.

A growing understanding of the cellular and molecular events that commonly occur during fibrosis in various tissues, including skeletal muscle, has provided a strong foundation for the development of effective therapies to prevent fibrosis and improve tissue healing. Because TGF-β1 plays such a crucial role in tissue fibrosis, particularly in skeletal muscle, it warrants attention as a key target for anti-fibrotic applications. Of the agents to block TGF-β1, Losartan potassium, a non-peptide molecule that works as an angiotensin II receptor blocker, is particularly attractive for clinical application as it is FDA approved and has minimal side effects. In a murine model, we have found that angiotensin receptor blocker-treated mice exhibited a histological, dose-dependent improvement in muscle regeneration and a significant reduction in fibrous tissue formation within the area of injury.

Given that Losartan has already been used clinically with an extremely safe side effect profile, we have conducted two case studies in young college athletes that sustained recurrent hamstring injuries and whose recoveries were safely improved with losartan. This is an off-label use of losartan (i.e.: the FDA has not approved labeling the device for the described purpose). Here we report the results obtained.

METHODS

Both subjects were submitted to the same protocol that is herein described: after obtaining the subjects' informed consent for treatment, both subjects were started on a 30-day treatment course of losartan at the manufacturer's recommended oral dose of 50 mg per day. Both subjects were healthy and had none of the contra-indications for the use of losartan. In addition to the medication, they underwent a routine rehabilitation program that gradually progressed to eccentric strengthening. The subjects reported no side effects while they were taking the study medication and remained normotensive throughout.

They were initially evaluated by clinical examination and were subsequently evaluated every 7 days with serial measurements (with a hand-held dynamometer - Lafayette Instrument Inc) of hamstring flexibility and strength as well as their blood pressure. Prior to the start of the medication the subjects were submitted to a magnetic resonance imaging (1.5T; GE-Sigma, Waukesha, WI, USA). After a period of 11 weeks the subjects underwent an isokinetic test (Biodex II) to better evaluate muscle strength compared to the non-injured side.

RESULTS

- Subjects

Subject #1: male, 21 years old, college athlete (football punter). He presented 10 days after an acute onset of "searing" pain in his left posterior thigh when he was kicking with his left leg. He referred a similar injury 5 weeks prior to the present injury.

Subject #2: male, 22 years old, college athlete (Ultimate Frisbee). He presented 4 days after an acute onset of pain in his left posterior thigh while he was sprinting. He referred two previous hamstring injuries (2 and 7 months prior to the present injury).

- MRI results (at time of injury)

Subject #1: Acute Grade 2 hamstring strain was observed with a partial thickness tear of the biceps femoris at the proximal myotendinous junction with surrounding edema without an associated avulsion fracture or hematoma.

Subject #2: Grade 2 strain with partial thickness tear of the left biceps femoris at the mid aspect, extends approximately 6 cm in the craniocaudal dimension.

- Hamstring flexibility and strength

Subject #1: By the third week after the injury, no deficit was evident in hamstring flexibility. By the ninth week, the isometric hamstring strength measurements at 30 and 90 degrees of knee flexion were 92 and 84% than the uninjured side respectively (Fig.1).

Subject #2: Also, by the third week after the injury, no deficit was evident in hamstring flexibility. By the ninth week, the injured side had a higher isometric hamstring strength measurement at 30 and 90 degrees of knee flexion. They were 132% and 110% than the uninjured side respectively (Fig.1).

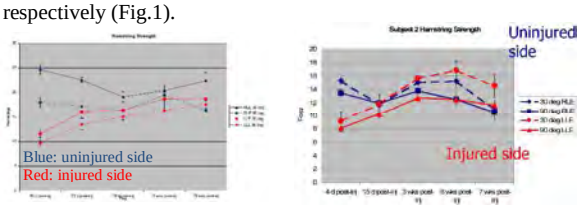

Figure 1: Isometric Hamstring strength (Kg)

- Isokinetic testing results

Subject #1: Eleven weeks after the injury, an isokinetic test of the hamstrings was performed showing an essentially normal result. Peak hamstring torque, on the uninjured side, was 96% of the injured side at 60 degrees per second and 107% of the injured side at 180 degrees per second.

Subject #2: Thirteen weeks after the injury, an isokinetic test of the hamstrings was performed showing an essentially normal result. Peak hamstring torque of the injured side was 96.3% compared to the uninjured side at 60 degrees per second and 97.3% of the uninjured side at 180 degrees per second.

DISCUSSION / CONCLUSION

We have described use of losartan, which is an FDA-approved angiotensin II receptor blocker, to treat two healthy collegiate athletes with a grade 2 biceps femoris injury. The patients tolerated the course of losartan well with no hypotension or any other side effects. Additionally, the patients demonstrated recovery of normal flexibility and strength compared to the contra-lateral leg. Both subjects were ready for return to sports in 9 to 11 weeks after injury.

ACKNOWLEDGEMENTS

This work was supported by the grants of the Henry J. Mankin Endowed Chair in Orthopaedic Surgery at the University of Pittsburgh and by the Albert B. Ferguson, Jr. MD Orthopaedic Fund of The Pittsburgh Foundation.

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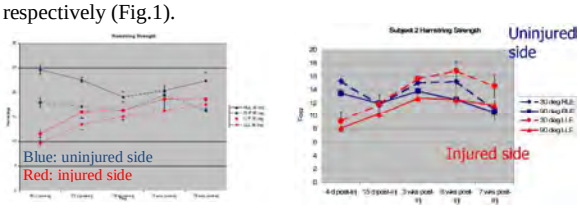

Figure 1: Isometric Hamstring strength (Kg)

- Isokinetic testing results

Subject #1: Eleven weeks after the injury, an isokinetic test of the hamstrings was performed showing an essentially normal result. Peak hamstring torque, on the uninjured side, was 96% of the injured side at 60 degrees per second and 107% of the injured side at 180 degrees per second.

Subject #2: Thirteen weeks after the injury, an isokinetic test of the hamstrings was performed showing an essentially normal result. Peak hamstring torque of the injured side was 96.3% compared to the uninjured side at 60 degrees per second and 97.3% of the uninjured side at 180 degrees per second.

DISCUSSION / CONCLUSION

We have described use of losartan, which is an FDA-approved angiotensin II receptor blocker, to treat two healthy collegiate athletes with a grade 2 biceps femoris injury. The patients tolerated the course of losartan well with no hypotension or any other side effects. Additionally, the patients demonstrated recovery of normal flexibility and strength compared to the contra-lateral leg. Both subjects were ready for return to sports in 9 to 11 weeks after injury.

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Musculoskeletal Pathology

Follistatin Improves Skeletal Muscle Healing after Injury and Disease through an Interaction with Muscle Regeneration, Angiogenesis, and Fibrosis

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Recovery from skeletal muscle injury is often incomplete because of the formation of fibrosis and inadequate myofiber regeneration; therefore, injured muscle could benefit significantly from therapies that both stimulate muscle regeneration and inhibit fibrosis. To this end, we focused on blocking myostatin, a member of the transforming growth factor–β superfamily and a negative regulator of muscle regeneration, with the myostatin antagonist follistatin. *In vivo*, follistatin-overexpressing transgenic mice underwent significantly greater myofiber regeneration and had less fibrosis formation compared with wild-type mice after skeletal muscle injury. Follistatin's mode of action is likely due to its ability to block myostatin and enhance neovacularization. Furthermore, muscle progenitor cells isolated from follistatin-overexpressing mice were significantly superior to muscle progenitors isolated from wild-type mice at regenerating dystrophin-positive myofibers when transplanted into the skeletal muscle of dystrophic mdx/severe combined immunodeficiency mice. *In vitro*, follistatin stimulated myoblasts to express MyoD, Myf5, and myogenin, which are myogenic transcription factors that promote myogenic differentiation. Moreover, follistatin's ability to enhance muscle differentiation is at least partially due to its ability to block myostatin, activin A, and transforming growth factor–β1, all of which are negative regulators of muscle cell differentiation. The findings of this study suggest that follistatin is a promising agent for improving skeletal muscle healing after injury and muscle diseases, such as the muscular dystrophies. ***(Am J Pathol 2011, 179:915–930; DOI: 10.1016/j.ajpath.2011.04.008)***

Although skeletal muscle injuries are extremely common, accounting for up to 35% to 55% of all sports-related injuries, the treatments that are currently available have not progressed during the last few decades and are often ineffective. Unfortunately, significant morbidity is associated with these injuries, such as the development of painful contractures, loss of muscle extensibility and strength, and the increased risk for repeated injury, which is largely due to extensive fibrosis formation. In response to traumas and disease, the local secretion of transforming growth factor (TGF)-β1, a potent fibrotic cytokine, induces the formation of fibrosis in various tissues and organs, including skeletal muscles.[1–9] Various agents, including suramin,[10,11] interferon-γ,[12] decorin,[5,8,13–15] relaxin,[16,17] and losartan,[9,18] have been shown to significantly enhance skeletal muscle regeneration, reduce fibrosis in injured muscles, and, in a broad spectrum of myopathic diseases, partially block TGF-β1. Although

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much of the pathogenesis after skeletal muscle injury has been attributed to TGF-β1 expression, it has become clear that myostatin, a member of the TGF-β superfamily, can also be implicated in the formation of muscle fibrosis.[19–24] Myostatin was initially known as a primary negative regulator of the growth and development of fetal and postnatal skeletal muscle.[25,26] A variety of approaches to block myostatin function have been developed during the past few years, including i) the creation of a myostatin gene knockout animal model, ii) the use of a myostatin neutralizing antibody, and iii) the delivery of the myostatin propeptide (*MPRO*) gene via an adenoassociated virus (AAV). These different methods of myostatin blockade have unequivocally shown that the inhibition of myostatin reduces fibrosis and enhances muscle regeneration in both injured and dystrophic murine skeletal muscles.[19–24] Myostatin directly stimulates the formation of skeletal muscle fibrosis by stimulating muscle fibroblasts, whose excessive activities are responsible for the development of fibrosis in injured muscle.[23,27] Muscle fibroblasts express the myostatin protein[23,27] and its receptor, ACVR2B.[27] Myostatin increases the proliferation and secretion of extracellular matrix products by muscle fibroblasts.[23,27] These effects may be due to the activation of the canonical TGF-β signaling pathway, as well as the PI3K/Akt/mTOR pathway in muscle fibroblasts, as evidenced by increased phosphorylation of SMAD2/3 and Akt/mTOR, respectively.[27] Injection of myostatin-coated beads into skeletal muscle leads to the formation of fibrosis around the injected beads, which could be reversed with the addition of follistatin, an antagonist of myostatin.[27] Myostatin and TGF-β1 have been observed to reciprocally induce the expression of one another.[23,28] The blockade of TGF-β1 signaling impairs myostatin's biological activity and vice versa, which suggests that TGF-β1 acts synergistically with myostatin to induce fibrosis in injured skeletal muscle.[23]

In addition to impairing skeletal muscle healing by promoting fibrosis, myostatin also inhibits myofiber regeneration in mouse models that mimic diseases such as Duchenne muscular dystrophy (DMD)[22] and amyotrophic lateral sclerosis–associated muscular atrophy,[29] as well as after experiencing a traumatic injury.[20,23] Specifically, the diaphragm muscles of mdx mice, an animal model of DMD, were noted to undergo significantly more myofiber damage and less myofiber regeneration when compared with transgenic mdx mice that also had their myostatin gene knocked out.[22] Similarly, in two acute muscle injury models, the first where the tibialis anterior muscle was injured by the injection of notexin and the other where the gastrocnemius muscle (GM) was injured via laceration, there was significantly greater regeneration and significantly less fibrosis formation in the injured myostatin knockout mice than the injured wild-type (WT) controls.[20,23] Given the promising benefits of blocking myostatin in skeletal muscle, a safety trial using MYO-29, a neutralizing antibody of myostatin, was conducted in adult patients with various forms of muscular dystrophy, including Becker muscular dystrophy, facioscapulohumeral dystrophy, and limb-girdle muscular dystrophy.[30] The results of this trial demonstrated that the patients could tolerate MYO-29 very well when it was administrated systemically.[30]

Research into the development of therapies to antagonize myostatin has led to the discovery of several new functions exhibited by follistatin. Follistatin was originally found to antagonize activin A in reproductive tissues and was also observed to neutralize several other proteins within the TGF-β superfamily.[31–33] Follistatin is also well known as a potent myostatin antagonist in skeletal muscle.[34,35] Follistatin-overexpressing transgenic mice exhibit a significant increase in muscle mass, much as is seen to occur in myostatin knockout mice.[35] Several *in vivo* studies on follistatin have shown that the systemic administration of this agent directly inhibits myostatin and also reduces myostatin-induced muscle wasting.[26,34,36] Moreover, a single injection of AAV-mediated follistatin into the quadriceps and tibialis anterior muscles, of both young and aged WT C57BL/6J or dystrophic mice, increased the muscles weight, and more interestingly, it also promoted an increase in the weight of noninjected muscles located remotely (eg, triceps muscles). This increase in muscular weight was accompanied by an increase in hind limb grip strength. It is also noteworthy that increased follistatin levels were not detrimental to the reproductive capacity of the treated animals.[37] Apart from these animal model findings, it has also been reported that follistatin plays a beneficial role in human myoblast transplantation. Human myoblasts-overexpressing follistatin outperformed normal human myoblast controls in both proliferation and differentiation capacities *in vitro* and regenerated much larger engraftment areas when injected into the tibialis anterior muscles of severe combined immunodeficiency (SCID) mice.[38] The safety and effectiveness of follistatin treatment have been evaluated in nonhuman primates.[39] The long-term expression of the AAV-mediated follistatin in the quadriceps muscles of cynomolgus macaque monkeys increased the monkeys' muscle mass and strength without having any deleterious effects on any of their critical organ systems.[39] This minimal off-target effect makes this molecule a promising potential therapeutic agent to treat muscles injured acutely and injured by degenerative muscle disorders; however, before translating follistatin-based therapies from the bench to the bedside, clear mechanisms of how follistatin promotes muscle regeneration requires extensive investigation.

In this report, we provide *in vivo* and *in vitro* data to support the application of follistatin as a potential therapeutic agent to enhance skeletal muscle healing after injury and disease. In addition, we investigated the underlying mechanisms of action that follistatin has on muscle cell regeneration, angiogenesis, and fibrosis formation. Specifically, we show that follistatin-overexpressing transgenic mice undergo more efficient skeletal muscle regeneration while developing less fibrosis after muscle injury (laceration) compared with WT controls. This enhancement of muscle healing in follistatin-overexpressing mice after injury appears to be related, at least in part, to an increase in the myogenic potential of muscle progenitor cells (MPCs), likely due to follistatin's inhibition of myostatin, activin A, and TGF-β1. Finally, we

also performed experiments to determine how follistatin affects the expression and downstream signaling of TGF-β1 and the expression of a variety of myogenic transcription factors.

Materials and Methods

Animal Model

Comparison of Muscle Healing between WT and Follistatin-Overexpressing Mice after Injury

All animal experiments were approved by the Children's Hospital of Pittsburgh's Institutional Animal Care and Use Committee. We performed partial cross-sectional lacerations on the GMs of 23 male C57BL/6 WT (Jackson Laboratories, Bar Harbor, ME) and 23 male follistatin-overexpressing mice (all mice were 7 to 8 weeks of age) according to a previously published protocol.[11–14,17,23] The muscles were then harvested at 1 (n = 3), 2 (n = 8), and 4 (n = 8) weeks and at 1.5 years (n = 4) after creating the laceration injury. Each of the harvested muscles had the percentage of fibrosis and muscle regeneration quantified, as previously described.[23] Briefly, after the cryosectioning of these muscles, histologic staining was performed with a Masson's trichrome kit (IMEB Inc., Chicago, IL). The amount of fibrosis formation in each of the muscles was measured by selecting three representative and nonadjacent sections and photographing up to three microscopic fields (×20). Images were taken for each section to ensure that the entire muscle section was completely photographed. We then pieced the images together in Adobe Photoshop CS3 (San Jose, CA) and quantified the percentage area of fibrosis using Northern Eclipse software version 6.0 (Empix Imaging Inc., Cheektawaga, NY) by measuring the area of fibrotic tissue along the sites of injury and then dividing this area by the total cross-sectional area of the entire tissue section.

To evaluate skeletal muscle regeneration, we stained sections from each harvested muscle with H&E. For each sample, three nonconsecutive sections were chosen in each section and images were taken from two to five microscopic fields (×100) and then pieced together, as described above, to make sure the entire injured area was covered. The smallest diameters of centronucleated myofibers, which represent newly regenerating muscle fibers, were quantified with Northern Eclipse software. This technique of measuring the smallest diameters of the centronucleated myofibers is a widely used method for evaluating muscle regeneration.[22,23,26,37,40,41] The diameters of more than 350 nonconsecutive, centronucleated myofibers were measured in each of the GMs. Moreover, the percentages of the regenerating myofibers were also determined in each of the injured muscles at all time points.

In addition, we performed immunohistochemical (IHC) staining to detect myostatin, activin A, collagen type IV, and phosphorylation of SMAD2/3 in each of the injured GMs. We also stained for CD31, which is an endothelial cell marker that we used as an index of neoangiogenesis in the injured muscles, via IHC staining. The CD31-positive structures were counted using Northern Eclipse software.

Intramuscular Injection of AAV2-MPRO/GFP into WT Mice

Twenty male C57BL/6J WT mice (8 weeks old; Jackson Laboratories) were used for these experiments. Fifty microliters of AAV serotype 2 (AAV2)–MPRO (2.5×10^{12} viral genome/mL) was injected into both GMs of 10 mice. The same dose of AAV2-GFP was injected into the GMs of 10 additional mice as controls. One month after AAV2 vector injection, both GMs of each mouse were subjected to laceration injury. The mice were sacrificed 4 weeks after creating the laceration injury, and muscle regeneration and fibrosis were measured as described previously for the follistatin-overexpressing and WT mice.

Fluorescence Immunostaining

Frozen GMs were sectioned at a thickness of 10 μm, and IHC analysis was then performed to detect myostatin, activin A, collagen type IV, and phosphorylation of SMAD2/3. Tissue sections were fixed in 4% formalin for 5 minutes followed by two 10-minute washes with PBS. The sections were first incubated with 10% horse serum (HS; Vector Laboratories, Burlingame, CA) for 1 hour to block nonspecific staining. Goat antimyostatin, activin A (R&D Systems, Minneapolis, MN), p-SMAD2/3 (Santa Cruz Biotechnology Inc., Santa Cruz, CA) or rabbit anticollagen IV (Meridian, Saco, ME) primary antibody were diluted 1:100 in 2% HS in PBS and incubated on the sections overnight at 4°C. Sections were then washed three times with PBS and incubated with a secondary antibody, anti-goat/rabbit IgG conjugated with biotin (1:200) (Vector Laboratories), for 1 hour at room temperature, followed by a PBS wash. Finally, streptavidin conjugated with Alexa Fluor 555 or 488 (1:500) (Invitrogen, Carlsbad, CA) was applied to each section for an additional hour. DAPI (Sigma, St Louis, MO) was used to counterstain the nuclei. We then quantified the amount of myostatin expression that was present in the injured muscles using Northern Eclipse software, which measures the area and intensity of the detected antibody of interest's signal along the sites of injury. This was then divided by the total cross-sectional area of the tissue to calculate the percentage of the injured muscle tissue that was positive for the detected antibody of interest. The negative controls were stained using the same procedure but without the primary antibody.

To monitor angiogenesis, we first incubated the sections with 10% HS for 1 hour and then incubated with a rat CD31 primary antibody (BD Biosciences, San Jose, CA) that was diluted 1:150 in 2% HS in PBS. This preparation was incubated for 1 hour at room temperature. The sections were then washed three times with PBS and incubated for 30 minutes with the secondary antibody, rabbit anti-rat IgG conjugated with Alexa Fluor 555 (Invitrogen). Finally, DAPI was used to counterstain the nuclei.

MPC Isolation and Transplantation into Skeletal Muscle

Using a modified preplate technique,[42,43] we isolated a fraction of MPCs with properties of low adhesion to collagen and long-term proliferation. Briefly, we determined the sex of the neonatal mice by anatomically sexing them and then isolated five populations of WT MPCs from five male neonatal C57BL/6J mice and also isolated seven populations of follistatin-overexpressing MPCs from seven male neonatal follistatin-overexpressing mice. These cells were expanded in proliferation medium consisting of Dulbecco's modified Eagle's medium (Invitrogen), 10% HS (Invitrogen), 10% fetal bovine serum (Invitrogen), 1% penicillin-streptomycin (Invitrogen), and 0.5% chicken embryo extract (Accurate Chemical & Scientific Corporation, Westbury, NY).

After the MPC populations were expanded *in vitro*, MPCs were injected into the GMs of female mdx/SCID mice. These mice were generated by crossbreeding mdx (C57BL/10ScSn-Dmdmdx) and SCID (C57BL/6J-prkdcscid/SzJ) mice (Jackson Laboratory) at our institution's animal facility. Approximately 3×10^5 cells from each cell population were transplanted into the GMs of four female mdx/SCID mice. All mdx/SCID mice were sacrificed 2 weeks after transplantation. The recipient GMs from these mice were harvested at this time, snap-frozen, and cryosectioned at a later date at a thickness of 10 μm per section.

Each section was immunostained for dystrophin with a rabbit anti-mouse dystrophin antibody to monitor the number of dystrophin-positive myofibers formed by the donor MPCs. The tissue sections were first fixed in 5% formalin for 5 minutes, followed by two 10-minute washes with PBS. The sections were blocked with 10% donkey serum for 1 hour and then incubated overnight at 4°C with a rabbit dystrophin primary antibody (Abcam, Cambridge, MA) that was diluted 1:500 in 5% donkey serum in PBS. The following day, these sections were washed three times with PBS and incubated with the secondary antibody donkey anti-rabbit IgG conjugated with Alexa Fluor 594 (Invitrogen). Images (×200) of the sections representing the largest dystrophin-positive engraftment areas in up to 10 microscopic fields were taken for each sample and then spliced together to cover the entire engraftment area. Dystrophin-positive myofibers were then counted to assess the efficiency of cell transplantation in the skeletal muscle of the mdx/SCID mice.

Flow Cytometry

To characterize WT and follistatin-overexpressing MPC populations, we used flow cytometry using antibodies specific for CD34 and Sca-1 on both WT and follistatin-overexpressing MPCs to analyze the percentage of stem cells in the MPC populations as previously described.[42,43] Briefly, cultured cells were trypsinized, centrifuged, and washed twice with PBS. We subsequently resuspended our cell pellets, blocked them with 10% mouse serum (Sigma) for 10 minutes on ice, and then applied rat anti-mouse monoclonal conjugated antibodies (CD34 phycoerythrin, Sca-1 allophycocyanin; BD Biosciences) and incubated on ice for 30 minutes. After this incubation period, we excluded nonviable cells by adding 7-aminoactinomycin D (BD Biosciences) to each sample. Cells were then evaluated with a FACS Caliber flow cytometer (Becton Dickinson) and analyzed with CellQuest software (Becton Dickinson).

Cell Culture

A C2C12 myoblast cell line (ATCC, Manassas, VA) was seeded overnight onto collagen-coated 12-well plates in normal growth medium (10% fetal bovine serum and 1% penicillin-streptomycin in Dulbecco's modified Eagle's medium). The next day, this medium was replaced with low-serum medium (2% HS and 1% penicillin-streptomycin in Dulbecco's modified Eagle's medium), which promotes the myogenic differentiation of myoblasts, supplemented with different combinations of the recombinant proteins outlined below. The medium and recombinant proteins were changed every 48 hours.

In the first set of experiments, C2C12 myoblasts were stimulated with follistatin recombinant protein (Sigma-Aldrich, St. Louis, MO) and cultured for up to 4 days. The effect of follistatin on myogenic differentiation and the expression of MyoD, Myf5, myogenin, and myostatin were examined by myosin heavy chain (MyHC) immunostaining and Western blot analysis.

In the second set of experiments, C2C12 myoblasts were stimulated with a combination of follistatin, activin A, and myostatin. The myogenic differentiation of these cells was then evaluated via MyHC immunostaining by calculating the fusion index (ie, the ratio of nuclei in myotubes to all nuclei) according to a previously published protocol.[23] Images of five representative microscopic fields were taken of each well from the 12-well plates.

In the final set of experiments, we incubated C2C12 myoblasts with varying concentrations of the recombinant proteins follistatin and TGF-β1. The cells were then either cultured for 4 days and stained for MyHC to check their myogenic differentiation capacity or cultured 16 hours before collecting the cells to analyze them for TGF-β1, SMAD2, and p-SMAD2 expression. In the latter set of cells, cell lysates were collected in preparation for Western blot analysis.

Western Blot

Cultured cells were lysed with T-PER Tissue Protein Extraction Reagent with the addition of protease inhibitors (Pierce, Rockford, IL). Equal amounts of cellular protein were loaded into each well and separated by 10% SDS-PAGE. Nitrocellulose membrane blotting was then performed under standard conditions. For immunoblotting, we used the following primary antibodies: mouse anti-β-actin IgG (1:8000) (Sigma-Aldrich), rabbit anti-TGF-β1 IgG (1:1000) (Abcam Inc.), goat anti-Myf-5 (Santa Cruz Biotechnology Inc.), mouse anti-MyoD and myogenin IgGs (1:250) (BD Biosciences), and goat anti-SMAD2 and p-SMAD2 IgGs (Cell Signaling Technology, Danvers, MA).

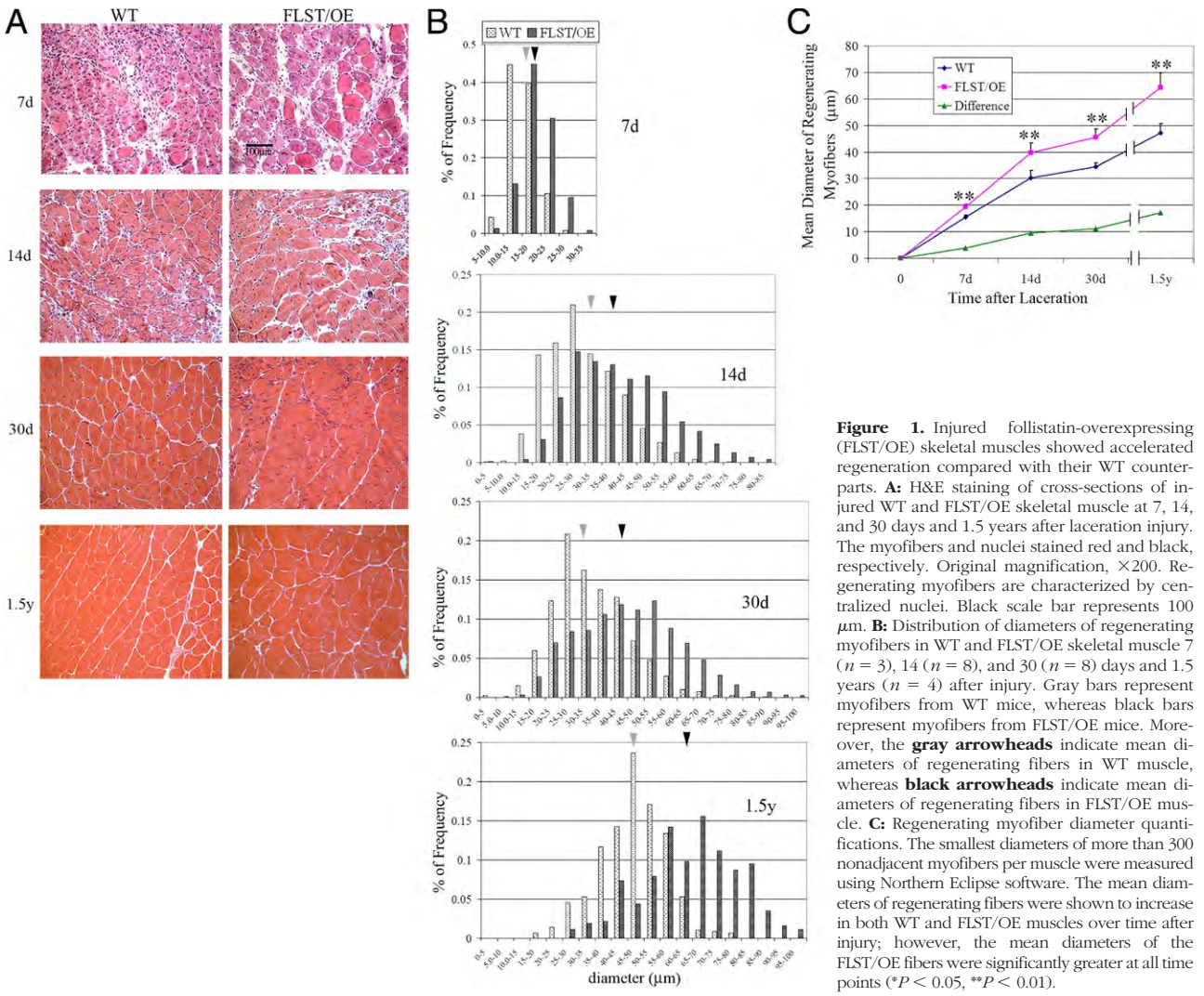

Figure 1. Injured follistatin-overexpressing (FLST/OE) skeletal muscles showed accelerated regeneration compared with their WT counterparts. **A:** H&E staining of cross-sections of injured WT and FLST/OE skeletal muscle at 7, 14, and 30 days and 1.5 years after laceration injury. The myofibers and nuclei stained red and black, respectively. Original magnification, ×200. Regenerating myofibers are characterized by centralized nuclei. Black scale bar represents 100 μm. **B:** Distribution of diameters of regenerating myofibers in WT and FLST/OE skeletal muscle 7 ($n = 3$), 14 ($n = 8$), and 30 ($n = 8$) days and 1.5 years ($n = 4$) after injury. Gray bars represent myofibers from WT mice, whereas black bars represent myofibers from FLST/OE mice. Moreover, the **gray arrowheads** indicate mean diameters of regenerating fibers in WT muscle, whereas **black arrowheads** indicate mean diameters of regenerating fibers in FLST/OE muscle. **C:** Regenerating myofiber diameter quantifications. The smallest diameters of more than 300 nonadjacent myofibers per muscle were measured using Northern Eclipse software. The mean diameters of regenerating fibers were shown to increase in both WT and FLST/OE muscles over time after injury; however, the mean diameters of the FLST/OE fibers were significantly greater at all time points (*$P < 0.05$, **$P < 0.01$).

Real-Time PCR

C2C12 cells were grown in media containing varying amounts of follistatin at 0, 200, 400, and 1000 ng/mL that was added every 48 hours and collected at 1 and 4 days after plating using Trizol (Invitrogen). RNA was precipitated, processed, and purified using the Qiagen RNeasy kit. RNA was DNase treated and quantitated on a spectrophotometer. To normalize variation among samples, a standard amount of 10 ng of RNA was used. Promega Go-TaqII-step real-time quantitative PCR (A6010) was used to run all samples. Predesigned SYBR primer sets were obtained from Qiagen for both myostatin and glyceraldehyde-3-phosphate dehydrogenase. The ABI 7900HT fast real-time PCR system was used to obtain C_T values of the genes of interest. C_T values were calculated using ABI SDS 2.3 software (Applied Biosystems by Life Technologies, Carlsbad, CA). Samples were normalized to glyceraldehyde-3-phosphate dehydrogenase expression, and the $\Delta\Delta C_T$ method of data analysis was used. The formula for fold change is $2^{-}(\Delta\Delta C_T)$ and is used to show differences among treatments. Gene expression levels were examined and normalized to untreated control cultures.

Statistical Analysis

All data are reported as mean ± SD or mean ± SEM, and data analyses have been performed with Student's *t*-test for comparisons between two groups and with a one-way analysis of variance for comparisons among three or more groups (SPSS Inc., Chicago, IL). For all statistically significant differences observed after performing one-way analysis of variance, the appropriate multiple comparison tests were used to perform a post hoc analysis. Statistical significance was considered at $P < 0.05$.

Results

Improved Healing in Follistatin-Overexpressing Skeletal Muscle

To investigate whether follistatin could promote skeletal muscle healing after injury, we used a follistatin-

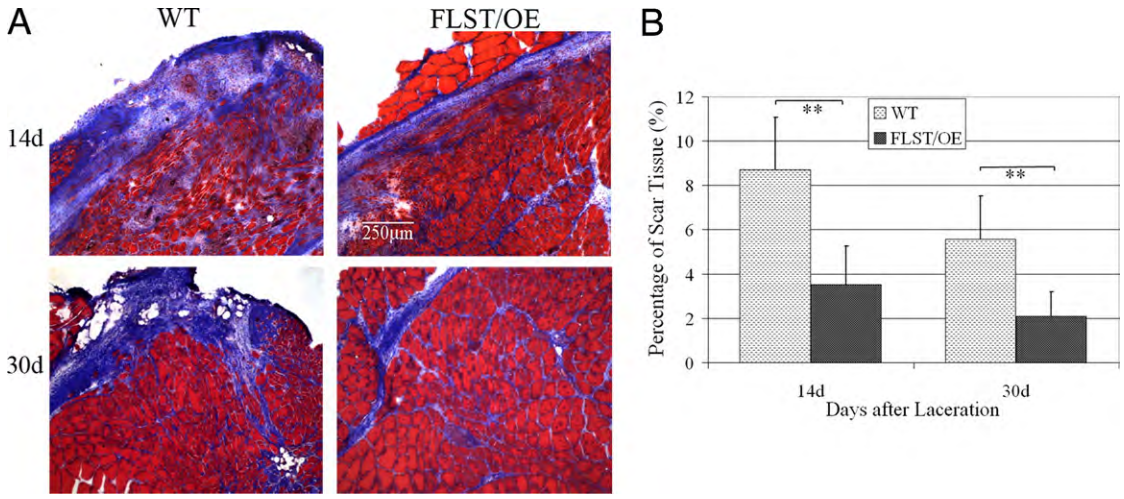

Figure 2. Fibrosis formation in the injured follistatin-overexpressing (FLST/OE) muscle was reduced when compared with the injured WT muscle. Masson's trichrome staining was performed on sections of injured FLST/OE and WT muscle (myofibers in red; fibrosis in blue). **A:** Representative images of injured FLST/OE and WT muscle at 14 ($n = 8$) and 30 ($n = 8$) days after injury. Original magnification, ×100. There was significantly less fibrosis observed in the injured FLST/OE muscles than the WT muscles. **B:** Injured FLST/OE muscles developed significantly less fibrosis than did injured WT muscles (**$P < 0.01$).

overexpressing transgenic mouse model to examine whether skeletal muscle healing after injury would differ from WT controls. After laceration, the GMs, from the WT and follistatin-overexpressing mice, were both observed to undergo regeneration as confirmed by the presence of centronucleated myofibers at the site of injury (Figure 1A). The myofiber diameters were determined 7 days after laceration and ranged in size from 5 μm to 35 μm (Figure 1B). Over time, the diameters of the regenerating myofibers increased in size, with the mean diameter of the regenerating myofibers in the follistatin-overexpressing muscle being significantly larger than those observed in the WT muscle. The mean diameters of the regenerating follistatin-overexpressing myofibers, as measured at the postlaceration time points of 7, 14, and 30 days and 1.5 years, were larger by approximately 25.3% (19.37 ± 0.80 μm versus 15.46 ± 0.81 μm; $P < 0.01$), 31.6% (39.77 ± 3.69 μm versus 30.22 ± 2.75 μm; $P < 0.01$), 32.5% (45.55 ± 3.03 μm versus 34.38 ± 1.56 μm; $P < 0.01$), and 36.3% (64.36 ± 5.40 μm versus 47.22 ± 3.49 μm; $P < 0.01$), respectively, than those of the WT mice (Figure 1C). The absolute differences in the mean diameters of the regenerating myofibers between the WT and follistatin-overexpressing mice were also shown to increase (green curve in Figure 1C). Accordingly, the injured muscles of follistatin-overexpressing mice compared with the WT animals contained a higher percentage of larger myofibers at each of the postinjury time points tested. For example, at 7 days 51% of the regenerating WT myofibers were larger than 15 μm, and 86% of the regenerating follistatin-overexpressing myofibers were larger than 15 μm. At 14 days, 45% of the WT regenerating myofibers were larger than 30 μm, whereas 73% of the follistatin-overexpressing regenerating myofibers fell into the 30- to 85-μm diameter group. At 30 days, 40% of the regenerating WT myofibers were larger than 35 μm, whereas 73% of the regenerating follistatin-overexpressing myofibers were larger than 35 μm (Figure 1B). Nevertheless, no significant difference was found in the percentage of regenerating myofibers between the injured follistatin-overexpressing muscles and the WT control muscles (see Supplemental Figure S1 at *http://ajp.amjpathol.org*).

In addition to the differences in myofiber regeneration, we observed significant differences in the deposition of collagenous connective tissue after injury between the regenerating WT and follistatin-overexpressing muscles. Specifically, at 14 days after laceration, fibrosis developed extensively in the WT muscles but was relatively limited in the follistatin-overexpressing muscles (Figure 2A). The amounts of fibrosis quantified at 14 days after injury within the WT and follistatin-overexpressing GMs, respectively, were 8.71% ± 2.36% and 3.54% ± 1.71% ($P < 0.01$) (Figure 2B). Compared with these values obtained at 14 days, our quantification analysis showed a reduction in fibrosis at 30 days in the injured WT and follistatin-overexpressing GMs (Figure 2A). Despite this, the relative amounts of fibrosis formation in the WT GMs continued to be significantly greater than the follistatin-overexpressing GMs (5.57% ± 1.94% versus 2.10% ± 1.10%; $P < 0.01$) (Figure 2B). Fibrosis in the injured GMs of the WT and follistatin-overexpressing mice was reduced at 1.5 years after injury (data not shown).

It has been reported that follistatin inhibits myostatin by directly binding to the molecule.[34,35] Notably, we found a reduction of the myostatin protein in the injured muscles of follistatin-overexpressing mice. This became apparent 2 weeks after injury, when we noted that the follistatin-overexpressing muscles showed less myostatin signal than the WT muscles, as indicated by immunostaining (Figure 3A). Collagen IV (green) stains the basal lamina, which outlines the myofibers, and the myostatin protein (red) is seen to be mostly localized in the cytoplasm of the regenerating, centronucleated fibers (Figure 3A, arrows); however, some of the myostatin-positive regenerating myofibers lacked a com-

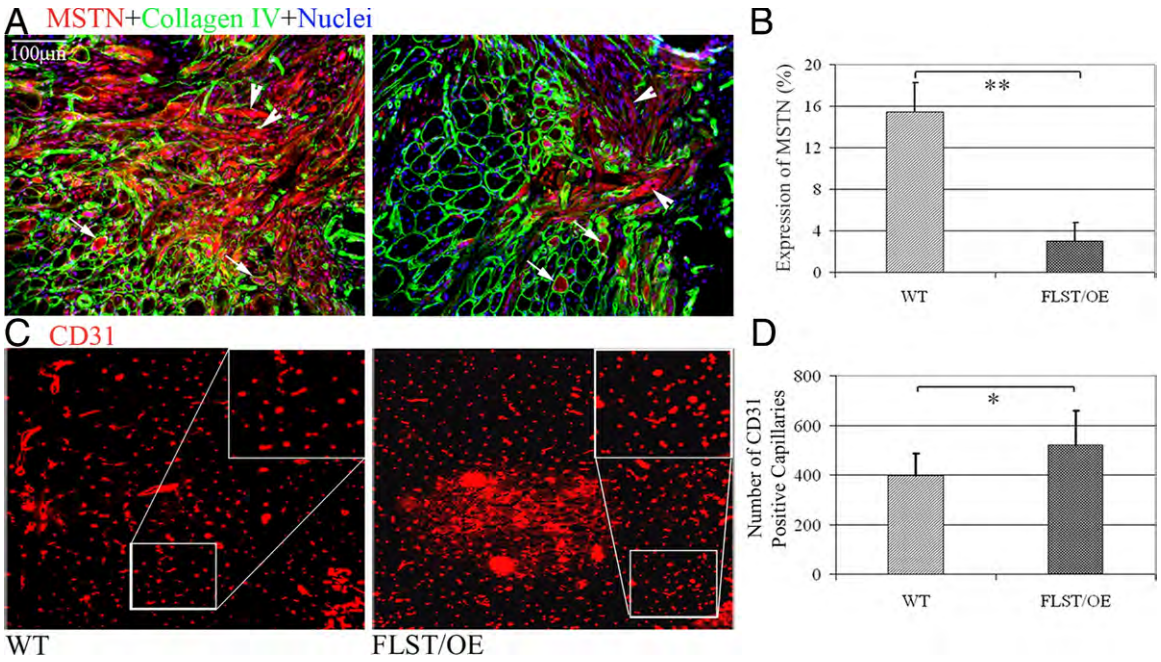

Figure 3. Decreased myostatin (MSTN) expression and increased angiogenesis in injured follistatin-overexpressing (FLST/OE) skeletal muscles. **A:** IHC analysis was performed to detect MSTN (red) and collagen type IV (green) expression in injured WT and FLST/OE muscle. Collagen type IV was used to highlight the basal lamina of myofibers, including necrotic, intact, and regenerating myofibers. MSTN-positive signals were seen within some of the regenerating myofibers surrounded by the basal lamina (**arrows**) and some expression outside the basal lamina (**arrowheads**). Original magnification, ×200. Injured FLST/OE muscles contained less MSTN staining than did injured WT muscles. **B:** When we measured the relative MSTN positive signals and areas, we found that there was significantly more MSTN expression detected in the injured WT muscles than in injured FLST/OE muscles. **C:** CD31, an endothelial cell marker, was used to stain capillary-like structures in the injured muscles. Original magnification, ×200. **D:** There were a significantly greater number of $CD31^+$ signals present in the injured FLST/OE muscles than in the injured WT muscles ($n = 8$) ($^*P < 0.05$, $^{**}P < 0.01$).

pletely intact basal lamina (Figure 3A, arrowheads). When measuring the area and intensity of the myostatin signal in the injured muscles, we found that myostatin in the injured follistatin-overexpressing muscles was significantly lower than that observed in the WT controls (Figure 3B). Similarly, 2 weeks after injury, the activin A immunostaining signal in the injured follistatin-overexpressing muscles was also decreased when compared with the WT controls (see Supplemental Figure S2 at *http://ajp.amjpathol.org*). Furthermore, we detected a trend that phosphorylation levels of SMAD2/3 in the injured muscles of follistatin-overexpressing mice were lower than that observed in the WT controls (see Supplemental Figure S3 at *http://ajp.amjpathol.org*).

It has been found that follistatin stimulates angiogenesis both *in vitro* and *in vivo*[44] and that the presence of increased angiogenesis has been reported to be involved with a reduction in the formation of fibrosis.[45] These findings have led us to examine neovascularization in the injured follistatin-overexpressing and WT muscles. We assessed the vascularity of injured follistatin-overexpressing and WT muscles at 30 days after laceration and observed that the follistatin-overexpressing muscles had a significantly larger number of $CD31^+$ capillary-like structures along the zone of injury compared with their WT counterparts (Figure 3, C and D). This finding indicates that increased vascularity may be, at least partially, responsible for the improved muscle healing observed in follistatin-overexpressing mice.

Comparison of WT and Follistatin-Overexpressing MPCs

To elucidate why follistatin-overexpressing muscles show a better regenerative capacity after injury than WT muscles, we compared MPCs isolated from follistatin-overexpressing skeletal muscle to WT MPCs. We used flow cytometry and immunocytochemistry to analyze the expression of stem cell (Sca-1, CD34) and myogenic (desmin) markers and also determined the proliferation and myogenic differentiation capacities of these two cell populations. The heterogeneous profile of the stem cell marker expression is apparent from the histograms in Figure 4A. Cell populations from both the follistatin-overexpressing and WT groups expressed these surface markers ($Sca\text{-}1^+$ and $CD34^+$, $Sca\text{-}1^+$ only, and $CD34^+$ only), although at different levels. Compared with the WT MPC populations, the follistatin-overexpressing populations contained a significantly larger percentage of cells that were positive for Sca-1 (Figure 4, A and B). The representative images of the flow cytometry dot plots showed that one of the follistatin-overexpressing MPC populations and one of the WT MPC populations contained 46.5% and 24% $Sca\text{-}1^+$ cells, respectively (Figure 4C). In the low serum medium, both follistatin-overexpressing and WT MPCs were capable of fusing into multinucleated myotubes as demonstrated by MyHC and DAPI staining (Figure 4D). The percentage of desmin-positive cells and proliferation and myogenic differentiation capacities were also compared between the follista-

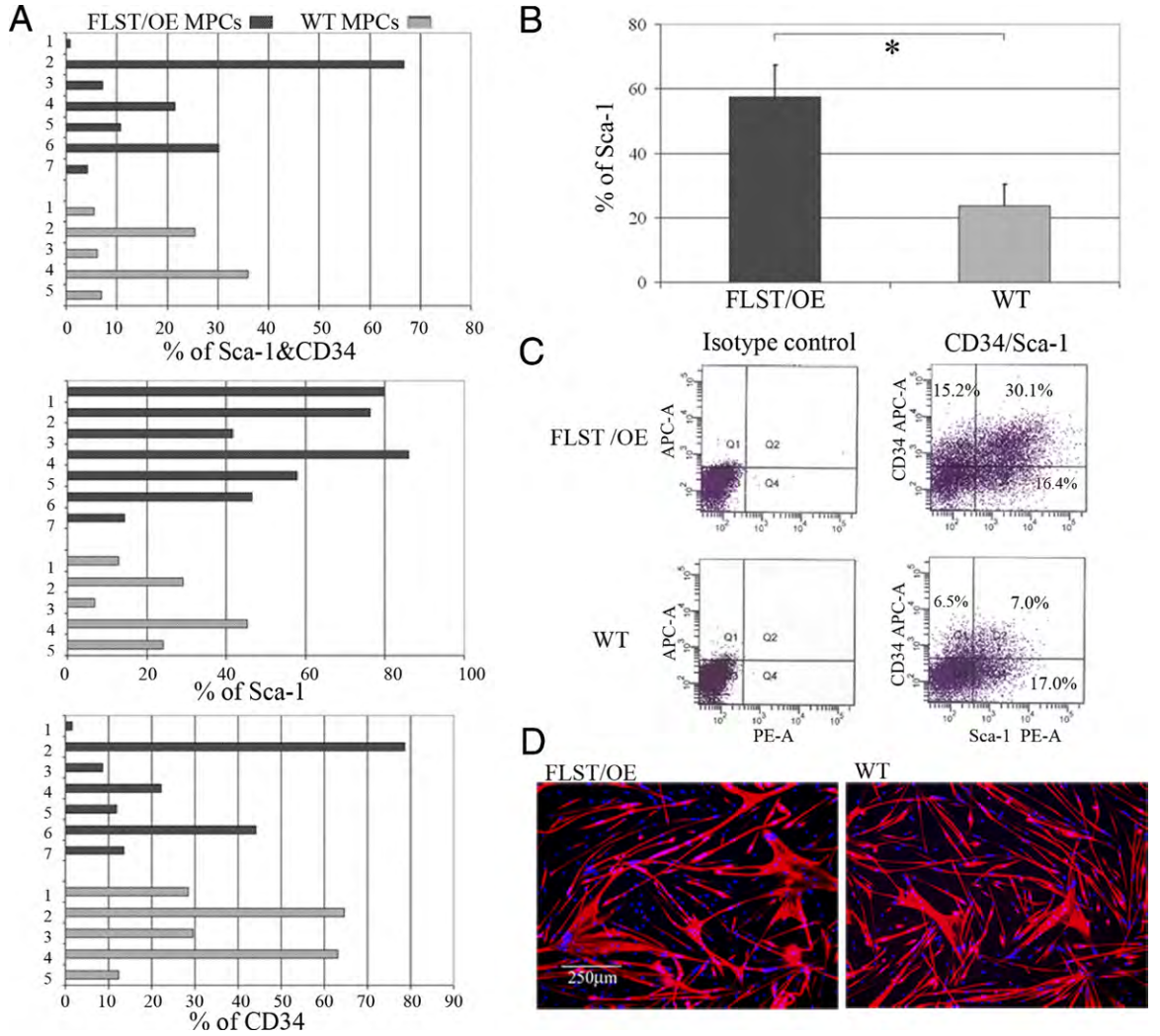

Figure 4. Characterization of MPCs. Seven follistatin-overexpressing (FLST/OE) and five WT MPC populations were examined for Sca-1 expression, CD34 expression, and *in vitro* myogenic differentiation. **A:** Histograms showing wild variability in the percentages of Sca-1$^+$ and CD34$^+$, Sca-1$^+$, and CD34$^+$ expressing cells among the MPC populations. **B:** Quantitation revealed a significant increase in the Sca-1$^+$ fraction in the FLST/OE MPC populations compared with the WT MPC populations. **C:** Images on the **left** are isotype controls; images on the **right** are representative images of a flow cytometry dot plot showing that FLST/OE MPC populations consist of a larger proportion of Sca-1$^+$ cells than the WT MPC populations (46.5% versus 24%). APC-A indicates allophycocyanin-area; PE-A, phycoerythrin area. **D:** Both FLST/OE and WT MPC populations underwent myogenic differentiation as labeled by MyHC (red) and DAPI (blue). Original magnification, ×100.

tin-overexpressing and WT MPC populations; however, because of high variability, no significant differences were observed (data not shown).

MPCs are a population of primary, long-term proliferating cells that regenerate skeletal muscle more efficiently than myoblasts when transplanted into the muscles of dystrophic mice.[43,46] We posited that follistatin-overexpressing MPCs would likely be superior to WT MPCs at regenerating skeletal muscle because of our finding that injured follistatin-overexpressing muscles underwent better muscle regeneration than did the WT control muscles. Using the preplate technique,[43,46] we isolated MPCs from both WT and follistatin-overexpressing mice and compared their ability to regenerate skeletal muscle by injecting them into the GMs of mdx/SCID mice. Quantification of the number of dystrophin-positive myofibers was performed to evaluate the cell transplantation efficiency. Notwithstanding, a high degree of variability was observed in the WT and follistatin-overexpressing MPCs' abilities to regenerate myofibers *in vivo* (Figure 5A). Only two of five of the WT MPC populations produced more than 200 dystrophin-positive myofibers, whereas six of seven of the follistatin-overexpressing cell populations regenerated more than 200 myofibers. Specifically, whereas the WT MPC population with the greatest potential to generate dystrophin-positive myofibers was able to regenerate 400 fibers, four of the seven follistatin-overexpressing populations regenerated between 500 and 1398 fibers (Figure 5A). Moreover, all of the follistatin-overexpressing MPC populations produced more fibers than the mean fiber number (eg, 195.6 myofibers) produced by the WT MPC populations (Figure 5, A and B). Overall, the follistatin-overexpressing MPCs (n = 7) produced significantly larger muscle engraftments than did the WT cells (n = 5) (592.8 ± 154.9 versus 195.6 ± 65.4; P = 0.023; Student's t-test) (Figure 5B). Two representative dystro-

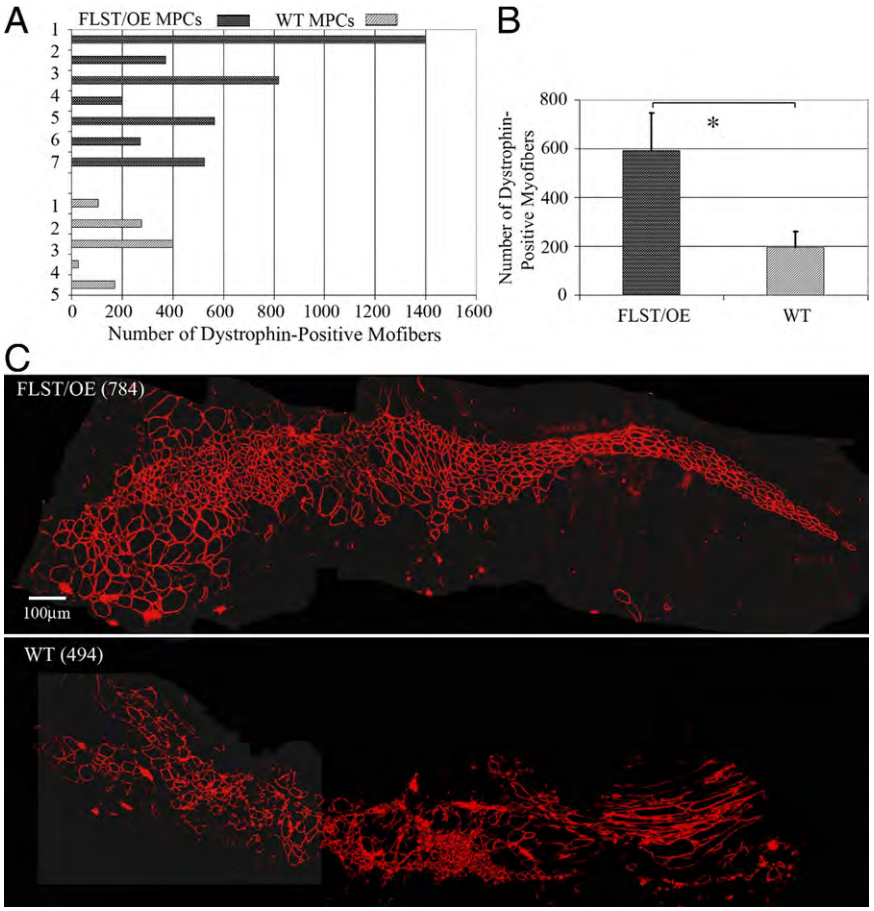

Figure 5. Follistatin-overexpressing MPCs (FLST/OE MPCs) regenerated skeletal muscle more efficiently than WT MPCs, when transplanted into the GMs of mdx/SCID mice. **A:** Quantitation of engraftment in terms of the number of dystrophin-positive fibers regenerated by the FLST/OE and WT MPC populations. **B:** The overall mean ± SEM number of dystrophin-positive myofibers was significantly greater for the FLST/OE MPCs (592.8 ± 154.9; $n = 7$ FLST/OE MPC populations; four muscles per population) than for the WT MPC populations (195.6 ± 65.4; $n = 5$ WT MPC populations; four muscles per population; $^{**}P = 0.023$, Student's *t*-test). **C:** Representative engraftments showed that the transplanted MPCs regenerated dystrophin-positive myofibers (red) within dystrophic muscle. FLST/OE MPCs produced more dystrophin-positive myofibers than did WT MPCs ($^{*}P < 0.05$, $^{**}P < 0.01$). Original magnification, ×200.

phin-positive engraftments derived from the follistatin-overexpressing and WT MPCs (784 versus 494 dystrophin-positive myofibers, respectively) are shown in Figure 5C.

Interaction between Follistatin and TGF-β Superfamily Members

Recombinant myostatin protein inhibited the myogenic differentiation ability of C2C12 myoblasts *in vitro*, and our results indicate that follistatin can neutralize exogenous myostatin, thereby allowing the C2C12 myoblasts to undergo myogenic differentiation (Figure 6A). We further showed that, much as is the case with myostatin's effect on myoblasts, activin A can also significantly inhibit the myogenic differentiation capacity of C2C12 cells (Figure 6B). We also demonstrated that follistatin can significantly attenuate the myogenic inhibitory effect that activin A has on C2C12 myoblast's capacity to differentiate into myotubes (Figure 6C).

Myogenic differentiation, in particular, involves sequential steps where myoblasts initially retract from the cell cycle and thereafter differentiate and fuse into multinucleated myotubes. TGF-β1 inhibits myoblast differentiation, and although muscle cells are able to withdraw from the cell cycle when cultured in fusion induction medium supplemented with TGF-β1, these cells fail to fuse into myotubes as is evidenced by the lack of muscle creatine kinase and nicotinic acetylcholine receptor expression.[47] Here, we have shown that TGF-β1 inhibits the myogenic differentiation of C2C12 myoblasts in fusion induction medium. In contrast to the extensive myotube formation observed in the control C2C12 cells not treated with TGF-β1, there were only a few small myotubes interspersed among numerous nuclei (blue) in cell cultures treated with TGF-β1 (Figure 7A). In cell cultures treated with both TGF-β1 and increasing concentrations of follistatin, follistatin was observed to counteract TGF-β1's inhibition of the C2C12 myoblast's myogenic capacity (Figure 7A). The fusion indices indicated that follistatin significantly increased the myogenic differentiation capacity of the C2C12 myoblasts and that TGF-β1 significantly decreased their myogenic differentiation capacity. Moreover, follistatin prevented TGF-β1 from inhibiting myogenesis and partially restored the myogenic differentiation capacity of the C2C12 myoblasts exposed to TGF-β1 (Figure 7B). Follow-up experiments demonstrated that follistatin also decreased TGF-β1 expression by the C2C12 myoblasts with and without the exogenous application of TGF-β1 (Figure 7C). In addition, the TGF-β1 signaling pathway relies on the activation of an intracellular SMAD signaling cascade, and our results indicated that follistatin blocks this pathway

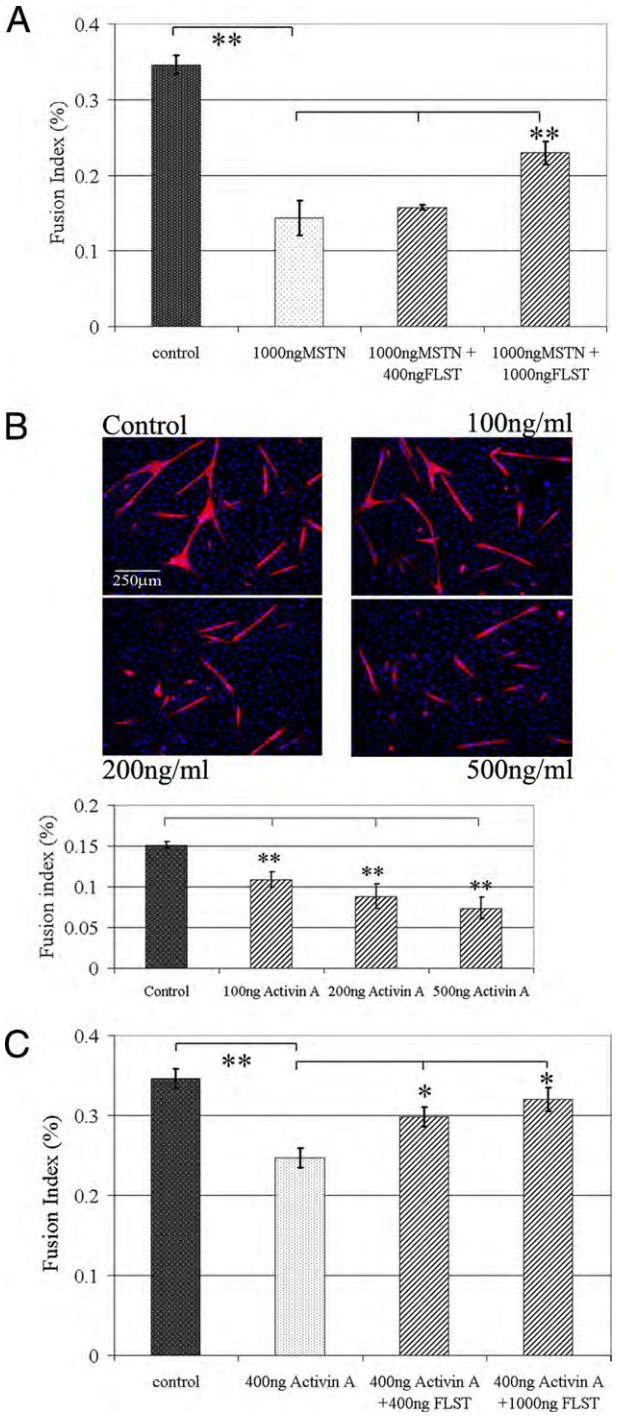

Figure 6. Interactions between follistatin (FLST) and myostatin (MSTN) and FLST and activin A. **A:** As indicated by the fusion index (the ratio of nuclei in myotubes to total nuclei), MSTN significantly inhibited C2C12 myoblast differentiation, but FLST counteracted MSTN and attenuated its inhibition of cellular differentiation. **B:** Without intervention, C2C12 myoblasts underwent myogenic differentiation in low serum medium as evidenced with MyHC (red) and DAPI (blue). Activin A significantly reduced muscle cell differentiation and the formation of myotubes in cell culture. Original magnification, ×100. **C:** FLST could neutralize the inhibitory effect of activin A on myoblast differentiation as shown by FLST induced-restoration of differentiation ($n = 3$; $^*P < 0.05$, $^{**}P < 0.01$).

by reducing the expression and phosphorylation of SMAD2 (Figure 7D).

Our group has previously shown that follistatin stimulates C2C12 myoblasts to undergo myogenic differentiation[23] and significantly increases the fusion index of the cells when compared with untreated controls and does so in a dose-dependent fashion.[23] In our complementary experiments from the current study, during the early stages of myogenic differentiation (day 1), when there is no detectable myotube formation, there is a notable increase in the expressions of MyoD and Myf5 in the C2C12 cells treated with follistatin when compared with untreated cells. During

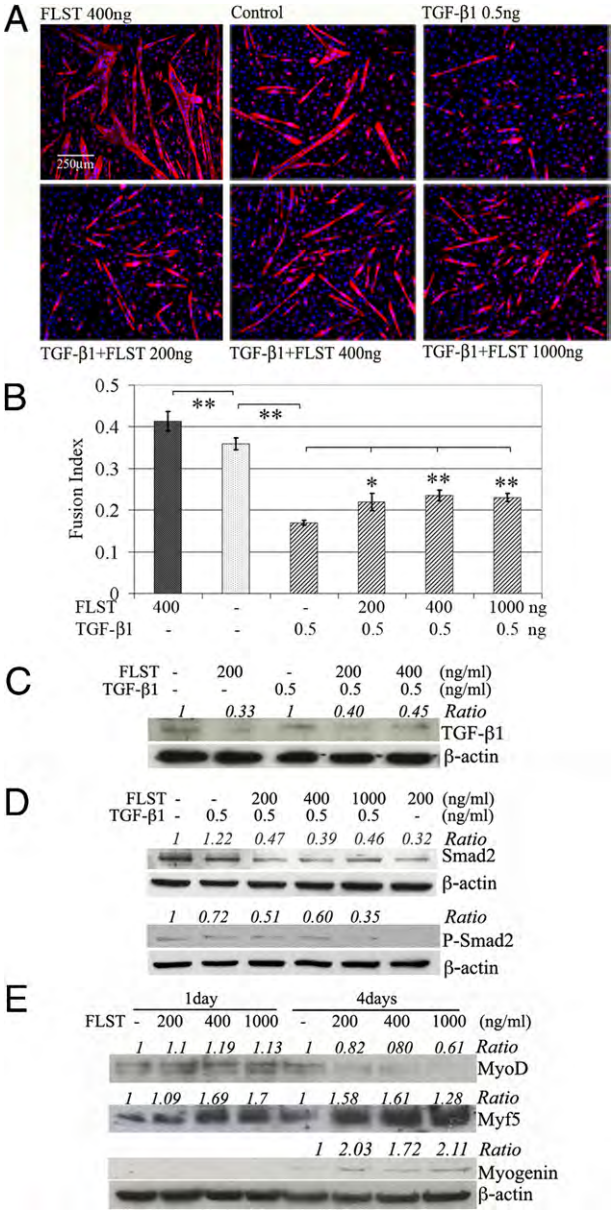

Figure 7. Follistatin (FLST) neutralized TGF-β1's activity on C2C12 myoblasts. **A:** Exemplary pictures of differentiation of C2C12 myoblasts treated by FLST alone, TGF-β1, and combinations of FLST and TGF-β1. Original magnification, ×100. Myotubes were visualized with MyHC (red) and DAPI (nuclei, blue). **B:** Our quantitative results showed that TGF-β1 significantly inhibited myogenic differentiation of C2C12 myoblasts. FLST was able to reverse TGF-β1–inhibited myogenic differentiation ($n = 3$). **C:** Western blot results showed that FLST decreased TGF-β1 expression in C2C12 myoblasts with or without the presence of exogenous TGF-β1. **D:** FLST also reduced the phosphorylation of SMAD2. **E:** FLST stimulated the expressions of the myogenic regulatory factors, MyoD, Myf5, and myogenin by myoblasts; the quantification of the Western blots was indicated as normalized ratio of proteins of interest to β-actin, whereas controls were referred to as 1 ($^*P < 0.05$, $^{**}P < 0.01$).

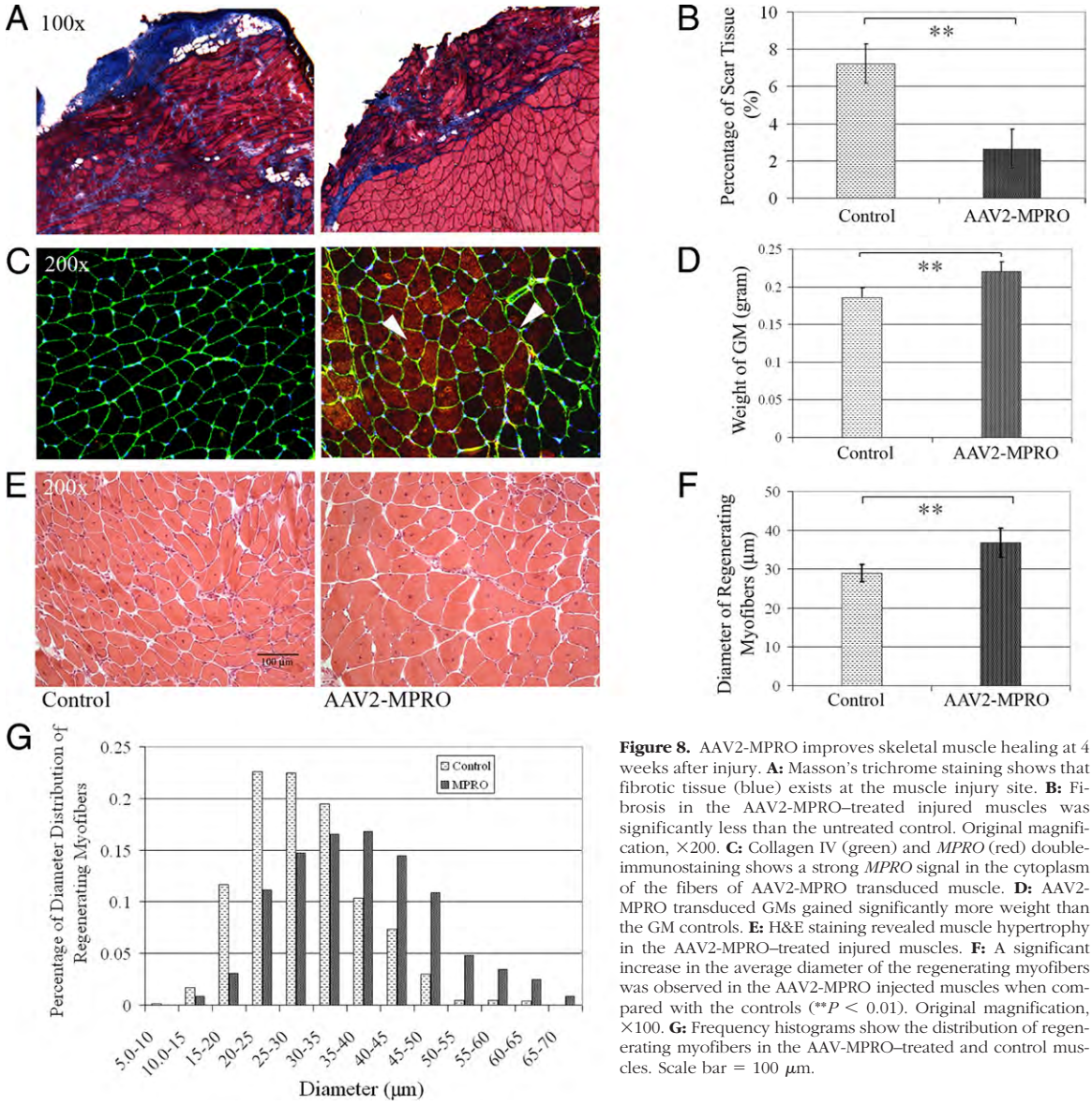

Figure 8. AAV2-MPRO improves skeletal muscle healing at 4 weeks after injury. **A:** Masson's trichrome staining shows that fibrotic tissue (blue) exists at the muscle injury site. **B:** Fibrosis in the AAV2-MPRO–treated injured muscles was significantly less than the untreated control. Original magnification, ×200. **C:** Collagen IV (green) and *MPRO* (red) double-immunostaining shows a strong *MPRO* signal in the cytoplasm of the fibers of AAV2-MPRO transduced muscle. **D:** AAV2-MPRO transduced GMs gained significantly more weight than the GM controls. **E:** H&E staining revealed muscle hypertrophy in the AAV2-MPRO–treated injured muscles. **F:** A significant increase in the average diameter of the regenerating myofibers was observed in the AAV2-MPRO injected muscles when compared with the controls (**$P < 0.01$). Original magnification, ×100. **G:** Frequency histograms show the distribution of regenerating myofibers in the AAV-MPRO–treated and control muscles. Scale bar = 100 μm.

the late stages of differentiation (day 4), in which the formation of myotubes is extensive, C2C12 cells exposed to follistatin augment their expression of myogenin and maintain elevated expression levels of Myf-5 compared with the untreated controls (Figure 7E).

AAV2-Delivered MPRO Inhibits MSTN Activity in Vivo

To make our results described above more therapeutically relevant, we performed an experiment to examine whether blocking MSTN using an AAV2-MPRO vector could improve the healing of injured skeletal muscle. Either an AAV2-MPRO or AAV2-GFP control vector was injected into the GMs of adult C57BL/6J mice 1 month before injuring the muscles. The mice were then sacrificed 4 weeks after GM laceration injury. Similar to what we observed in the follistatin-overexpressing muscles, the injured GMs overexpressing *MPRO* exhibited better healing than the AAV2-GFP transduced controls. Masson's trichrome staining showed extensive fibrosis infiltration in the injured WT skeletal muscle, whereas the injured AAV2-MPRO transduced muscle formed significantly less fibrotic tissue (Figure 8, A and B) (7.2% ± 1.1% versus 2.6% ± 1.0%; $P < 0.01$). The *MPRO* gene was stably expressed in the transduced muscles injected with the AAV-MPRO vector (Figure 8C). The weights of the AAV2-MPRO transduced GMs were also significantly greater than the WT counterpart muscles (Figure 8D). Moreover, we observed larger regenerating myofibers in the injured muscles transduced with AAV2-MPRO than in the injured WT muscles (Figure 8E). The mean diameter of the regenerating myofibers in the GMs overexpressing MPRO was significantly increased (by 26.7%) over what was observed in the nontransduced GMs (36.8 ± 3.8 μm versus 29.0 ± 2.2 μm; $P < 0.01$) (Figure 8F). Corre-

spondingly, the distribution of diameters of the regenerating myofibers revealed that 78.0% of the regenerating myofibers in the control muscles had diameters smaller than 35 μm when compared with 46.3% found in the AAV2-MPRO transduced muscles. In contrast, 53.7% of the regenerating myofibers in the injured AAV2-MPRO transduced muscles had a diameter in the range of 35 to 70 μm (Figure 8G).

Discussion

In this study, we showed that the skeletal muscle healing of follistatin-overexpressing mice is accelerated when compared with the skeletal muscle of WT mice. Specifically, the mean diameter of the regenerating myofibers in the injured follistatin-overexpressing muscles remained significantly larger than their WT counterparts. Moreover, fibrosis formation was significantly lower in the injured follistatin-overexpressing muscles than the injured WT muscles. These results are comparable to those that we previously observed in the injured GMs of myostatin knockout mice.[23] There are several possible explanations for follistatin's augmentation, which include i) decreasing levels of myostatin, activin A, and the phosphorylated SMAD2/3; ii) promoting vascularity in the injured muscles; and iii) enhancing the ability of MPCs to regenerate the injured muscle fibers. This last explanation was confirmed by *in vitro* results, which showed that follistatin could promote myoblast differentiation by blocking myostatin, activin A, and TGF-β1, all negative regulators of muscle regeneration, and also by augmenting the expression of the myogenic transcription factors MyoD, Myf5, and Myogenin. Although we discuss these events individually, we highlight that they are not mutually exclusive of one another but rather illustrate how follistatin can synergistically promote healing through each of these processes.

Mechanisms Involved in the Reduction of Fibrosis Formation in Injured Follistatin-Overexpressing Muscle

TGF-β1 expression in injured skeletal muscle is time dependent, peaking at 3 to 5 days and then again at 10 to 14 days after injury.[5,6,48] The latter event appears to be associated with the formation of fibrosis and ineffective muscle regeneration.[5] When the second peak of TGF-β1 expression is blocked with the administration of an antifibrotic agent at 14 days after injury, it leads to histologic and physiologic improvements of the injured muscles.[5,10–14,16,17] Coincidently, our *in vivo* studies showed significant decreases in myostatin immunostaining among the injured follistatin-overexpressing GMs 14 days after injury. Given our *in vitro* results that follistatin does not down-regulate the mRNA expression of myostatin in C2C12 myoblasts (see Supplemental Figure S4 at *http://ajp.amjpathol.org*), the decreased myostatin levels observed in the injured follistatin-overexpressing muscle may not be the result of a decrease in myostatin transcription. It has been shown that follistatin can be released into the blood circulation[26,37]; therefore, the reduction in the myostatin signal in the injured follistatin-overexpressing muscle, partially caused by excessive circulating follistatin and follistatin within the muscle's extracellular matrix, can sequester myostatin and prevent it from binding to the injured muscle tissue. Moreover, some small regenerating myofibers, without intact basal lamina, were found in the current study to be strongly myostatin positive in the injured WT and follistatin-overexpressing GMs, which was similar to our previous findings.[23] Li et al reported that some regenerating myofibers appeared to degrade and transform into myofibroblasts, which aggravated fibrosis formation in the injured skeletal muscles.[5] If this is the case, these myostatin-positive, basal lamina–deficient regenerating fibers may represent a transitional state of regenerating myofibers that are undergoing the differentiation process into myofibroblasts. A decrease in the amount of myostatin at the injury site likely accounts for the reduction in fibrosis observed in the injured follistatin-overexpressing muscles given the fact that myostatin directly stimulates fibrosis in skeletal muscle[20,23,27] and the lack of myostatin would also attenuate the profibrotic effects of TGF-β1.[23] In addition, we showed that follistatin down-regulates the expression of TGF-β1 and counteracts its activity *in vitro*. Myostatin, TGF-β1, and activin A all belong to the TGF-β superfamily and all signal through the TGF-β/SMAD2/3 signaling pathway. Our data indicate that there is an overall decrease in the phosphorylation level of SMAD2/3 in the injured follistatin-overexpressing muscle when compared with the WT controls. Although we do not have direct evidence showing that follistatin also reduces fibrosis through the inhibition of TGF-β1 and activin A, our results appear to demonstrate that follistatin decreases the TGF-β–like signaling that occurs through the SMAD2/3 pathway, thereby attenuating the inhibitory effect of this pathway on skeletal muscle healing.

We also showed that significantly more $CD31^+$ capillary-like structures appeared in the injured follistatin-overexpressing muscles than in the injured WT controls; however, more evidence is required to validate the vascular functionality of these $CD31^+$ structures. It is noteworthy to point out that angiogenesis correlates with an increase in skeletal and cardiac muscle regeneration and a reduction in fibrosis.[45,49–52] In the present study there is insufficient evidence to indicate whether follistatin directly stimulates angiogenesis in injured skeletal muscle; nevertheless, accumulating evidence in other studies suggests that follistatin expression can be positively correlated with angiogenesis by a variety of different cells and tissues.[42,53–55] Follistatin expression is up-regulated by activated endothelial cells but down-regulated on the cell's withdrawal from the cell cycle.[55] Follistatin promotes the proliferation of several types of endothelial cells, including human umbilical vein endothelial cells and bovine aortic endothelial cells.[55] Follistatin alone moderately stimulates angiogenesis in the rabbit cornea, and its angiogenic effect can be greatly reinforced when

combined with basic fibroblast growth factor.[55] In addition, follistatin was recently found to function as a binding protein of angiogenin, which is known to induce angiogenesis via the activation of endothelial and smooth muscle cells.[53] Follistatin and angiogenin colocalize in the nuclei of HeLa cells, suggesting that the follistatin and angiogenin interaction may play an important role in angiogenin-induced angiogenesis.[53] A "loss of function" experiment validated that the follistatin isoform 288 is essential for promoting angiogenesis during mouse embryo formation.[56] Importantly, activin A and TGF-β1 have been shown to inhibit the growth and activation of a spectrum of vascular endothelial cells[55,57]; therefore, it is possible that follistatin partially promotes angiogenesis by antagonizing TGF-β's inhibition of endothelial cells. Nevertheless, the effects that follistatin and TGF-β have on skeletal muscle vascular endothelial cells remain to be elucidated. Revascularization in injured skeletal muscle is a critical event during muscle regeneration. The speed and quality of tissue repair are directly correlated with the degree of vascular ingrowth into the muscle injury site, which provides an adequate supply of oxygen and nutrients to promote efficient regeneration.[58,59] The regeneration of multinucleated myofibers in injured muscles will be impaired if there is an insufficient supply of oxygen due to an inadequate supply of energy resulting from insufficient aerobic metabolism.[58,60] Given the stimulatory effects that follistatin exerts on endothelial cells, it is reasonable to speculate that follistatin may be able to promote the growth of new capillaries to increase the blood supply to the injury site. The increase in blood supply would further accelerate the resorption of scar tissue and increase muscle regeneration.

Cellular Mechanism by which Follistatin Promotes Skeletal Muscle Regeneration after Injury

The development of skeletal muscle during embryogenesis and its regeneration after trauma, or in the setting of skeletal muscle disease, largely occur through the differentiation of muscle cells into myofibers.[61–63] Satellite cells in adult skeletal muscle can be found within a niche between the basal lamina and sarcolemma of the myofibers, forming a pool of quiescent myogenic progenitor cells.[64] In response to muscle trauma and during disease processes, these cells are activated to reenter the cell cycle, migrate from the basal lamina to the zone of injury, and undergo asymmetric divisions. A preponderance of daughter cells are committed to differentiate and fuse into multinucleated myofibers, whereas a small portion of self-renewing cells replenish the reservoir of satellite cells by reentering a quiescent state.

Myostatin inhibits satellite cell self-renewal by downregulating their G1 to S progression within the cell cycle retaining the satellite cells in a quiescent state.[65,66] Inversely, myostatin knockout skeletal muscle possesses more satellite cells than their WT counterparts, likely resulting from an increase in proliferation and a delay in myogenic differentiation by the adult myostatin knockout satellite cells.[65] On the basis of this information, we sought to determine the impact that follistatin would have on the regenerative capacity of MPCs. MPCs are a heterogeneous population consisting of myoblasts, satellite cells, and stem cells. We injected MPCs isolated from both follistatin-overexpressing and WT skeletal muscle of mice into the GMs of mdx/SCID mice to determine their regeneration efficiency. We found that overall, the follistatin-overexpressing MPC populations regenerated a greater numbers of dystrophin-positive myofibers when compared with the WT MPCs; however, not all of the follistatin-overexpressing MPC populations outperformed their WT counterparts. Variations in regard to the regenerative capacity of both cell types was readily apparent and is a common phenomenon observed collectively when myoblasts, satellite cells, or muscle stem cells are transplanted intramuscularly.[43,46,67] Specifically, dystrophin-positive myofibers regenerated by the follistatin-overexpressing MPC populations varied from 200 to 1398 fibers, whereas those produced by the WT MPC populations varied from 27 to 400 fibers. The increase in the regenerative capacity of the follistatin-overexpressing MPC populations probably partially accounts for the better regeneration observed in the injured follistatin-overexpressing muscles than was seen in the WT muscles. These findings may help researchers to genetically engineer an optimal cell population for cell-based therapies to treat DMD, a lethal, sex-linked, recessive, muscle-wasting disease that stems from a variety of different mutations of the dystrophin gene.[68,69] For instance, the transplantation of muscle cells carrying a follistatin transgene into the dystrophic muscle of a DMD patient may enhance the success of cell transplantation compared with that of unengineered MPCs.[70]

We also investigated whether the cell surface marker profile, proliferation rate, and myogenic differentiation capacity of the MPCs could highlight the mechanism by which these cells display a higher muscle regenerative potential *in vivo*. We found that the MPC populations isolated from both types of mice exhibited a broad heterogeneity of cell surface markers, proliferation rates, and myogenic capacities *in vitro*; however, despite this heterogeneity, the follistatin-overexpressing MPC populations contained a significantly larger percentage of Sca-1$^+$ cells than the WT controls. It has been reported that among male muscle-derived stem cell populations, a higher percentage of Sca-1$^+$ cells in the cell population appeared to positively correlate with a higher regenerative capacity when transplanted into dystrophic mdx mouse skeletal muscle; however, this is not the case for female muscle-derived stem cells.[67] These findings are consistent with our current findings showing that all of the cells for these studies were isolated from male mice and the follistatin-overexpressing MPC populations used in the current study had a higher percentage of Sca-1$^+$ cells and showed a better regenerative capacity than the WT cells.

Molecular Mechanisms by which Follistatin Promotes Myogenesis: Interaction between Follistatin and TGF-βs

The overexpression of follistatin probably has a more profound effect on muscle growth than solely blocking myostatin. As stated above, we found that the differentiation of myoblasts *in vitro* is inhibited by myostatin, activin A, and TGF-β1. Each of these factors appears to be inhibited by follistatin; as a result, myoblast differentiation capacity can be restored. Although it has already been shown that follistatin directly antagonizes both myostatin and activin A, further investigation is required to determine whether follistatin inhibits TGF-β1 directly or indirectly. Moreover, evidence is emerging that activin A may be one of the key growth factors that function in tandem with myostatin to limit muscle growth.[41,71] The follistatin I mutant, which does not bind activin A effectively, promotes muscle growth to a lesser extent than WT follistatin. Furthermore, unlike WT follistatin, the mutant follistatin fails to induce an increase in the muscle mass of myostatin knockout mice.[71] It has been recently reported that activin A knockout mice exhibit significant size increases in both their pectoralis and triceps muscles, which provides genetic evidence that activin A may be one of the key growth factors that function in tandem with myostatin to limit muscle growth.[41] In this study, our results have shown that that follistatin inhibits not only myostatin but also activin A and TGF-β1 in C2C12 myoblast, which may partially explain why follistatin exerts a more striking effect in promoting muscle regeneration in both injured and dystrophic muscle.

There is some evidence, from previously published reports, that also indicates that follistatin's effect on muscle growth is not exclusively linked to blocking the effects of myostatin. First, follistatin-overexpressing mice display even greater skeletal muscle mass than do myostatin knockout mice, suggesting that follistatin's effect on skeletal muscle may result from the ablation of other negative regulators of muscle growth besides myostatin.[35] Second, Lee et al have also reported that the effect that follistatin has on skeletal muscle does not result solely from the inhibition of myostatin activity. Their findings demonstrated that the overexpression of follistatin led to further increases in the muscle mass of myostatin knockout mice when compared with myostatin knockout mice controls alone.[72] Third, blocking the activin type IIB receptor causes further increases in the skeletal muscle mass of myostatin knockout mice, suggesting that other growth factors can limit muscle growth via the activin type IIB receptor as does myostatin.[40] These results implicate the existence of other molecules that could be involved in the observed beneficial effects that follistatin has on muscle growth.

Skeletal Muscle Injury Comparisons between Myostatin Knockout and Follistatin-Overexpressing Mice

We have previously examined how the loss of myostatin benefits injured skeletal muscle using the same laceration injury model as in the current study.[23] When retrospectively comparing the current results with those collected from the myostatin knockout mice, we found that the mean diameter of the regenerating myofibers in the follistatin-overexpressing mice was 32.50% greater than in the WT mice 4 weeks after injury compared with a 21.22% increase over the WT controls seen in the myostatin knockout mice. Interestingly, there is not much difference in the formation of fibrosis between injured follistatin-overexpressing and myostatin knockout muscle. Although injured follistatin-overexpressing muscle shows more significant reductions in fibrosis formation than injured myostatin knockout muscle (59.36% verus 25.00%) 2 weeks after laceration, at 4 weeks after injury, these reductions in fibrosis were inconsequential between the two groups (66.70% verus 62.30%) (see Supplemental Table S1 at *http://ajp.amjpathol.org*). Similar reductions in fibrosis in injured myostatin knockout and follistatin-overexpressing muscle suggest that the inhibition of myostatin may largely account for the decrease observed in fibrosis in the injured follistatin-overexpressing muscles.

Myostatin knockout MPCs can regenerate significantly more myofibers than WT control MPCs when intramuscularly transplanted into mdx/SCID mice.[23] When follistatin-overexpressing, WT, and myostatin knockout progenitor cells were injected, in parallel, into the skeletal muscle of mdx/SCID mice, the follistatin-overexpressing MPCs regenerated three times as many dystrophin-positive myofibers as the WT MPCs (592.8 ± 154.9 versus 195.6 ± 65.4), whereas the myostatin knockout MPCs showed about a 1.5-fold increase over the WT MPCs (518.1 ± 117.6 versus 195.6 ± 65.4) (Figure 5, A and B; see also Supplemental Figure S5 and Supplemental Table S1 at *http://ajp.amjpathol.org*). Four of seven (57%) of the follistatin-overexpressing MPC populations regenerated more than 500 fibers; in contrast, only two of five (40%) of the myostatin knockout MPC populations did so (Figure 5A; see also Supplemental Figure S5 at *http://ajp.amjpathol.org*).

Apart from the results collected from the myostatin knockout and follistatin-overexpressing mice, we further demonstrated that AAV2-mediated *MPRO* gene therapy improved muscle healing in WT skeletal muscle by inhibiting myostatin. This finding suggests that inhibiting myostatin is applicable for the treatment of injured muscles and myopathic disorders. Taken together, follistatin-based therapies may represent a more effective therapeutic strategy over others that only antagonize myostatin. Moreover, because follistatin generally promotes muscle hypertrophy without targeting specific defective genes (eg, dystrophin, DMD), follistatin renders itself as a potential universal therapeutic reagent to treat a board spectrum of muscle diseases, especially the muscular dystrophies, which lack a clear pathogenic gene defect, such as facioscapulohumeral muscular dystrophy. Furthermore, follistatin exhibited no unusual adverse effects on the reproductive capacity of mice.[37] Although additional studies are required on primates to warrant follistatin's feasibility for clinical translation, its use stands to be

potentially beneficial for the treatment of a variety of skeletal muscle injuries and disorders.

Acknowledgments

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Angiotensin II Receptor Blocker and Muscle Derived Stem Cells Transplantation Treatment for Contusion Skeletal Muscle Injury in Mice

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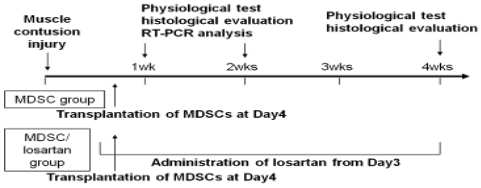
INTRODUCTION:

Muscle contusions are one of the most common muscle injuries in sports medicine. Although these injuries are capable of healing, incomplete functional recovery often occurs. We have previously reported that losartan, one of the FDA approved Angiotensin II Receptor Blockers (ARBs), can promote muscle healing by preventing fibrosis [1]. On the other hand, rapid revascularization after muscle injury is important for early muscle healing [2]. We have also previously shown that muscle derived stem cells (MDSCs) derived from blood vessels' cells have a multilineage differentiation potential that includes myogenic and endothelial lineages [3]. In the last ORS meeting, we reported that the better functional recovery after MDSC transplantation could be caused by increase in angiogenesis and decrease in fibrosis. However, our protocol for the MDSCs' transplantation could not completely inhibit fibrosis formation within the injured muscle. The aims of this study were 1) to find the effect of administration of losartan before MDSCs' transplantation and 2) to investigate the healing mechanism of MDSCs' transplantation with losartan treatment.

METHODS:

The contusion was performed on tibialis anterior (TA) muscle of C57BL/6 wild-type mice [4]. MDSCs were isolated from 3-week-old wild-type mice (C57BL/6J) using a preplate technique as previously described [5]. At 4 days post-injury, 3×10^5 MDSCs were transplanted directly into the injured TA muscle region (n = 15 mice for each group). Concentrations of LOS (125mg) in 1 liter of drinking water were administered beginning 3 days post-injury until endpoint. These doses were calculated based on the average fluid intake of mice as 10mg/kg/day. Mice were divided into 3 groups, 1) MDSC/losartan group, 2) MDSC group, and 3) injured control group with PBS injection and drinking normal water. At 1, 2, and 4 weeks post injury, a modified in situ force physiological testing was performed [6]. After the testing, animals were sacrificed and the TA muscles were harvested for histological evaluations. Statistical analysis was performed with ANOVA and Scheffe's F test as post hoc test. Statistical significance was defined as $p < 0.05$.

Figure 1.



RESULTS:

MDSCs' Transplantation Enhanced Muscle Regeneration in Injured Muscle: After hematoxlin and eosin staining, the centronucleated regenerating myofibers in the injured muscle were counted and compared among the groups at 2 weeks post-injury. The MDSC treated groups (MDSC and MDSC/losartan groups) showed a significantly higher number of regenerative myofibers (MDSC group, 131.8±32.1; MDSC/losartan group, 132.4±28.5/hpf) when compared with control group (40.7±36.2/hpf) (Fig. 2A).

Administration of Losartan Decreased Fibrosis Formation in Injured Muscle: After Masson's trichrome staining, the area of fibrotic scar tissue was evaluated and compared among the groups at 4 weeks post-injury. MDSC/losartan group showed significantly less fibrotic area (3.74±1.41%) when compared with control and MDSC groups (48.24±22.99 and 19.39±4.12%, respectively) (Fig. 2B).

MDSCs' Transplantation Showed Rapid Improvement of Muscle Strength: At 2 weeks post-injury, The MDSC treated groups (MDSC and MDSC/losartan groups) showed significantly greater specific peak twitch and tetanic forces (twitch; 44.1±10.3 and 47.2±13.7, tetanic; 67.2±16.2 and 69.4±13.1g/cm^2, respectively) when compared with control group (twitch; 24.9±5.3, tetanic; 46.8±11.6g/cm^2). Moreover, there was no significant difference between the MDSC treated groups and normal group (twitch; 36.0±11.1, tetanic; 64.9±18.8g/cm^2) (Fig. 3).

At 4 weeks after the injury, The control group showed significantly less specific twitch and tetanic forces (twitch; 13.8±2.0, tetanic; 17.4±2.2g/cm^2) when compared with the other groups (MDSC and MDSC/losartan groups) (twitch; 20.6±3.1 and 22.4±2.1, tetanic; 25.6±2.7 and 31.9±2.9g/cm^2, respectively). Interestingly, MDSC/losartan group showed a significantly greater specific peak tetanic force when compared with MDSC and normal (twitch; 20.0±0.8, tetanic; 24.1±0.8 g/cm^2) groups (Fig. 3).

MDSCs' transplantation after Losartan Treatment Enhanced FSTN and Reduced p-Smad2/3 in Injured Muscle: FSTN expression areas in the injured TA muscles were measured and compared among the groups at 1 week post-injury. MDSC/losartan group (0.60±0.22%) showed a significantly greater FSTN expression when compared with MDSC and control groups (0.29±0.19 and 0.00±0.00 %, respectively) (Fig. 4A). p-Smad 2/3 expression areas in the injured TA muscles were measured and compared among the groups at 2 weeks post-injury. MDSC/losartan group showed significantly lower p-Smad 2/3 (0.25±0.34%) expressions when compared with control and MDSC groups (22.5±13.4 and 8.29±5.02%, respectively) (Fig. 4B)..

Figure 2.

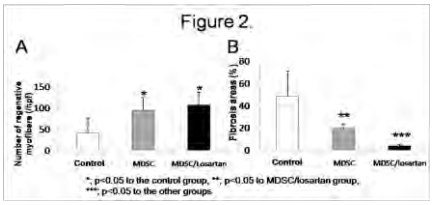
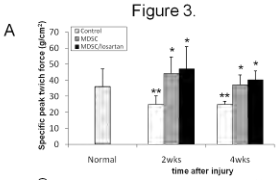


Figure 3.



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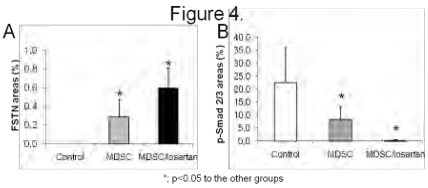

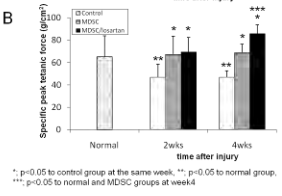

DISCUSSION:

The combination therapy (losartan with MDSCs therapy) following a contusion injury improved overall skeletal muscle healing. We observed a larger number of centronucleated myofibers, less area of fibrosis, and better functional recovery in the combination therapy group than in PBS and MDSC groups 4 weeks after injury. Functional recovery after skeletal muscle injury is the most important factor for clinical translation of this therapy. Additionally, in this combination therapy, the expression of p-Smad 2/3, which is a transcription factor that regulates the fibrosis, was lower than in the other groups 2 weeks after injury. These results suggest that the effect of losartan in MDSCs transplanted after skeletal muscle injury could contribute to the development of biological treatments to accelerate muscle healing.

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Characterization of Losartan's Mechanism of Action for Muscle Healing

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INTRODUCTION:
Muscle injuries are a very common musculoskeletal problem encountered in sports medicine. We have previously reported that Losartan (LOS), one of the Angiotensin II Receptor Blockers, when administered at a clinically equivalent dose for the treatment of hypertension in humans (10 mg/kg/day) 3 days after contusion injury, can accelerate muscle healing due to its antifibrotic effect on injured skeletal muscle [1]. LOS is an FDA approved antihypertensive medication and has a well-tolerated side-effect profile; however, its mechanism of action at the clinical dose on the muscle healing process remains unclear. The angiotensin II type 1 receptor (AT1) regulates the expression of myostatin (MSTN) which is a primary negative regulator of muscle growth and a strong stimulator of fibrosis formation, inflammation [2, 3] (figure 1). We hypothesized that regulating the expression of AT1 during the inflammation phase could accelerate muscle healing after skeletal muscle injury by balancing the beneficial aspects of the inflammation process. In this study, we investigated the expression of AT1, MSTN and MyoD (a regulator of muscle regeneration [4]) at different time points after contusion injury and at different initiation times of LOS administration (Day 0 group LOS started at the time of injury, Day 3 LOS started 3 days after injury).

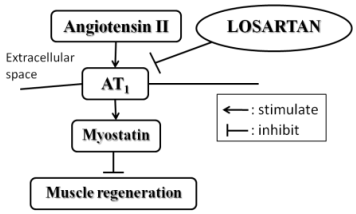

Figure 1. Signal pathway

METHODS:
Animal model: The muscle contusion model was developed in the tibialis anterior (TA) muscle of C57BL/6J wild-type mice. LOS administration, at the concentration of 125mg per 1 liter of tap water, was initiated on either day 0 or 3 post-injury and continued until endpoint dates. The control group was given drink tap water (6 mice per group). The dosing was calculated based on the average fluid intake of mice as 10 mg/kg/day respectively [1]. At 3, 7 and 14 days post-injury, we harvested the TA muscles of the mice for analysis of AT1, MSTN and MyoD expression (Figure 2).

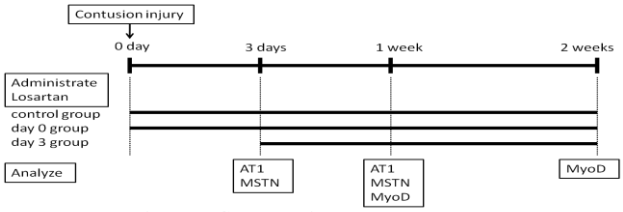

Figure 2. Study design

Evaluation of AT1, MSTN and MyoD Expression After LOS Therapy: The TA muscles were harvested, cryosectioned and immunohistochemically stained 3 days and 1 week after injury using antibodies against AT1 and MSTN. TA muscles were also harvested at 1 and 2 weeks after injury and were cryosectioned and immunohistochemically stained using an antibody against MyoD. The expression of AT1, MSTN and MyoD positive areas were calculated using Northern Eclipse software (Empix Imaging Inc).
Statistical analysis: Differences between samples were assessed by ANOVA and Scheffe's F test as a post hoc test. Statistical significance was defined as $p < 0.05$.

RESULTS:
LOS enhanced expression of AT1 (Figure 3): At 3 days post-injury, we observed significant increases in the day 0 group compared to the untreated control group. At 1 week after injury, the expression of AT1 in the day 3 group was significantly lower than the untreated control and day 0 treatment groups.
LOS enhanced expression of MSTN (Figure 4): The expression of MSTN expression in the day 0 group was significantly greater at 3 days after injury compared to the control group. At 1 week after injury, the expression of MSTN in the day 3 treatment group was significantly lower than the untreated control and day 0 treatment groups.
LOS enhanced expression of MyoD (Figure 5): At 1 week after injury, the expression of MyoD in the day 3 treatment group was greater than the untreated control and day 0 treatment groups (Figure 5A). Two weeks after injury, the expression of MyoD in the day 3 treatment group was also greater than the untreated control and day 0 treatment groups (Figure 5B).

DISCUSSION:
We demonstrated that the expression of MSTN and AT1 were significantly decreased when LOS was administered 3 days after injury when compared to untreated controls and in mice treated with LOS immediately following injury. Additionally the expression of MyoD in the day 3 group was increased at 1 and 2 weeks post-injury compared to the untreated control and day 0 treatment groups. It has been previously reported that LOS regulated the expression of MSTN via AT1 and that MSTN inhibits muscle regeneration [2, 3] (Figure 1). This suggests that regulating the expression of AT1 decreases the expression of MSTN, and then induces muscle regeneration, which was demonstrated by increasing MyoD expression 1 and 2 weeks after injury. These findings support our previously reported results that showed increased myofiber regeneration and physiological muscle force when LOS is administered 3 days after injury verses no treatment or immediate administration of LOS post-injury [1]. We therefore posit that the mechanism by which LOS accelerates the muscle healing is through the limited or later stage reduction of AT1 during the inflammation process after injury.

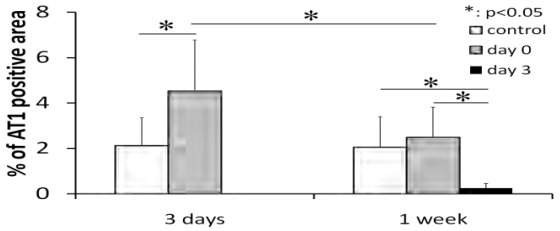

Figure 3. % of angiotensin II type 1 receptor (AT1) positive area

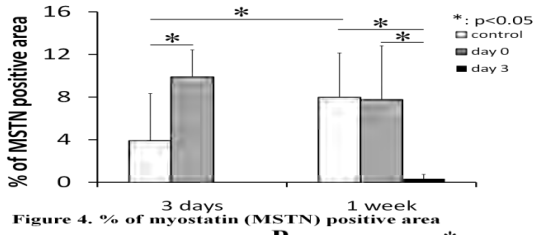

Figure 4. % of myostatin (MSTN) positive area

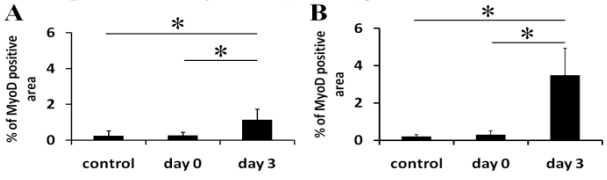

Figure 5. % of MyoD positive area
A; 1 week after injury. B; 2 weeks after injury.
*: p<0.05

ACKNOWLEDGEMENTS:
The authors are grateful for technical assistance from Jessica Tebbets, Michelle Witt, Ying Tang, Richardo Ferrari, Avidas Usas and Burhan Gharaibeh. Funding support was provided by a grant from the Department of Defense (W81XWH-06-1-0406) and AFIRM.

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Angiotensin II receptor blocker promote muscle healing after injury through activation of muscle regulations

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Our study revealed that the most effective timing for the administration of the human equivalent dose of losartan (10 mg/kg/day) was 3 days after muscle injury. The mechanism of muscle healing induced by the administration of losartan is not only due to a reduction of TGF-ß1 via the blockade of the AT1 receptors but is also due to the increased expression of follistatin.

INTRODUCTION:

Muscle injuries are very commonly encountered in sports medicine practice. Although muscle injuries are capable of healing, the complete functional recovery of the muscle is hindered by the formation of dense scar tissue which is triggered by TGF-β1 [1]. Losartan (LOS),

an angiotensin II receptor (AT1 receptor, which stimulate TGF-β1 etc.) blocker, is an FDA approved antihypertensive medication and has a very low side-effect profile and is well-tolerated. Our previous studies have revealed that 30 mg/kg/day of LOS treatment was effective at promoting muscle healing and reducing fibrosis formation in a murine model of skeletal muscle contusion injury [2,3]; however, this dose, which was administrated immediately after muscle injury, is higher than the recommended dose used in humans (10 mg/kg/day) to treat hypertension. We also investigated the administration of LOS three days after muscle injury and demonstrated muscle force recovery with the regeneration of muscle fibers and a reduction in the formation of fibrosis 4 weeks after injury [4]. Follistatin (FSTN) is a promising biological agent for improving skeletal muscle healing, and it well known that FSTN has been demonstrated to be a potent antagonist of TGF-β1 and another member of the ,TGF-β super family, myostatin (MSTN) [5]. MSTN is a primary negative regulator of the growth and development of fetal and postnatal skeletal muscle and is also a strong stimulator of fibrosis formation [6-8]. In the current study we investigated the use of LOS at the dosage used in human hypertensive patients (10 mg/kg/day) and analyzed the expression of FSTN and MSTN at different time points after contusion injury.

METHODS:

Animal model: A muscle contusion injury was created in the tibialis anterior (TA) muscle of C57BL/6J wild-type mice (8 weeks old, n=36). The administration of 125 mg of LOS

dissolved in 1 liter of drinking water was initiated on day 0 (Day 0 group, n=12) or 3 days after injury (Day 3 group, n=12) and continued for 1 or 2 weeks. This dose was calculated based on the average fluid intake of mice as 10 mg/kg/day. Drinking water not supplemented with LOS was administered to the animals in the untreated injury control group (control group, n=12). The LOS dosing was calculated based on the average fluid intake of mice [3]. One and two weeks after injury, we harvested the TA muscle for histology and western blot analyses.

Evaluation of FSTN and MSTN Expression After Losartan Therapy. The TA muscles were harvested 1 and 2 weeks after injury and were either cryosectioned and immunohistochemically stained using antibodies against FSTN and MSTN (Santa Cruz, Santa Cruz, CA), or the muscles had whole protein isolated and western blot analysis was performed to measure the FSTN and MSTN expression in the injured muscles. The expression of FSTN and MSTN positive areas in the immunohistochemical staining were calculated using Northern Eclipse software (Empix Imaging Inc). The FSTN and MSTN bands from the western blot were quantified using computerized densitometry (SCION Image, Scion Corporation, Maryland, USA).

Statistical analysis: The significance of difference was established by ANOVA followed by Scheffe's post hoc test. Statistical significance was defined as $p < 0.05$.

RESULTS:

LOS enhanced expression of Follistatin: Expression of FSTN detected by immunohistochemical staining indicated that FSTN and MSTN was significantly greater in the

Day 3 group than the control and Day 0 groups at 1 week after injury. At 2 weeks post-injury the expression of FSTN in the Day 3 group was significantly greater than in the control group. There was no significant difference between the three groups at the 1 and 2 week post-injury. The western blot analysis revealed the same results as the immunohistochemical staining assay revealed.

Reduction of Myostatin expression after LOS treatment: Expression of MSTN detected by immunohistochemical staining of the Day 3 group was significantly lower than in the control and Day 0 groups at 1 week after injury. Two weeks after injury there were no significant differences in the expression of MSTN between the groups; however the expression of MSTN in the control group 2 weeks after injury was significantly lower than at 1 week post-injury, which was also observed in the Day 0 group. The western blot analysis revealed the same results as the immunohistochemical staining assay revealed.

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DISCUSSION:

Our study revealed that the most effective timing for the administration of the human equivalent dose of LOS (10 mg/kg/day) was 3 days after muscle injury. In our previous study, we observed an increase in the number of centronucleated myofibers, a decrease in the area of fibrosis, and an enhancement in muscle strength when LOS was administered 3 days after muscle injury. Additionally, we have previously reported that muscle regeneration and fibrosis formation are

two concomitant processes that occur after a muscle injury is incurred, and that LOS has a greater effect on muscle regeneration than on preventing the formation of fibrosis [3]. Our current study demonstrated that the administration of LOS 3 days after injury leads to a reduction of fibrosis via the down-regulation of endogenous MSTN at 1 week after muscle contusion injury and an up-regulation of endogenous FSTN at 1 and 2 weeks post-injury. However, the mechanisms by which FSTN and MSTN influence muscle regeneration and repair is still unclear. We can therefore conclude that the mechanism of muscle healing induced by the administration of LOS is not only due to a reduction of TGF-β1 via the blockade of the AT1 receptors but is also due to the increased expression of FSTN.

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The Timing of Administration of a Clinically Relevant Dose of Losartan Influences the Healing Process after the Induction of a Muscle Contusion Injury.

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Running head: Angiotensin II receptor blocker therapy for muscle injury

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ABSTRACT: Losartan (LOS) is an FDA approved antihypertensive medication that has a well-tolerated side-effect profile. Our previous study revealed that the immediate treatment of a muscle injury with LOS was effective at promoting muscle healing and inducing an antifibrotic effect in a murine model of skeletal muscle injury. In the current study we first investigated the minimum effective dose of administering LOS immediately after injury and subsequently determined whether the timing of administration, of a clinically relevant dose of losartan, would influence its effectiveness for improving muscle healing after a contusion injury. In the first study, the mice were administered with 3, 10, 30 or 300mg/kg/day of LOS immediately after injury and the healing process was evaluated at 2 and 4 weeks post-injury. In the second study, 10mg/kg/day was administered immediately or at 3 or 7 days after injury and the healing process was then evaluated as described above. At 4 weeks post-injury, we observed a significant increase in muscle regeneration and a significant decrease in fibrosis, which consequently led to an improvement in muscle force in the 30 and 300mg/kg/day groups, when LOS was administered immediately following injury. We also observed a significant improvement in muscle healing at 4 weeks post-injury, when the clinically relevant dose of 10mg/kg/day was administered at 3 or 7 days after injury. Our study revealed accelerated muscle healing when the 30mg/kg/day and 300mg/kg/day of LOS was administered immediately after injury and when the clinically relevant dose of 10mg/kg/day of LOS was administered at 3 or 7 days post-injury.

Keywords: losartan, contusion injury, fibrosis, muscle regeneration,

INTRODUCTION:

Muscle injuries are a very common musculoskeletal problem encountered in sports medicine. In our laboratory, we have investigated several biological agents that provide benefit for accelerating the natural course of muscle injury. Specifically, we have focused our recent efforts on agents that inhibit muscle fibrosis via the inhibition of transforming growth factor-β1 (TGF-β1), a key cytokine in the fibrotic signaling pathway in skeletal muscle (24). Using decorin, suramin, relaxin, and gamma interferon, we have demonstrated that these therapies can decrease fibrosis and increase myofiber regeneration and the muscle's functional recovery, after skeletal muscle injury (4, 8-9, 25, 33); however, the clinical use of these antifibrotic agents, is hindered by several side-effects, the lack of oral dosing formulations, and in the case of decorin, suramin and relaxin, no approval by the Food and Drug Administration (FDA) for use in humans. Losartan (LOS), an angiotensin II receptor blocker (ARB), is an FDA approved antihypertensive medication and has a well-tolerated side-effect profile in humans. Our previous study using a murine model of skeletal muscle after injury revealed that LOS treatment was effective at promoting muscle healing through an antifibrotic effect (2). In the current study, we investigated the minimum dose of LOS required to improve muscle healing when administered immediately after injury. We also tested the use of a clinically relevant dose of LOS (10mg/kg/day) administered at different time points after muscle injury.

Myostatin (MSTN) is a highly conserved TGF-β superfamily member that is expressed in skeletal muscle (29). Deletion of the MSTN gene in mice leads to muscle hypertrophy and hyperplasia with an approximate doubling of muscle mass (29). It is well known that MSTN stimulates the proliferation of muscle fibroblasts and the production of extracellular matrix proteins both *in vitro* and *in vivo* (26). Moreover, in the absence/reduction of MSTN, muscles regenerate more quickly and completely following acute and chronic injury, including severe limb threatening conditions such as compartment syndrome (28, 44).

Follistatin (FSTN) on the other hand, which has several different isoforms and is expressed by almost all the tissues of the body (35), can inhibit several members of the TGF-β superfamily, including MSTN (1, 10). Indeed, over-expression of FSTN induces a dramatic increase in satellite cells and muscle mass when over-expressed in transgenic mice (23) or when delivered to the muscle via an adeno-associated virus (14)(10). In the current study we investigated the effect of the recommended clinically relevant human dose of LOS on the expression profiles of MSTN and FSTN when administrated at different time points after muscle contusion injury.

METHODS:

In Vitro Potential of Angiotensin II (ANGII) and LOS on C2C12 Myoblasts

Cell Culture --- C2C12 myoblast cells, a well-known myoblast cell line, were purchased from the American Type Culture Collection (Manassas, VA). C2C12 cells

were plated at a density of $1.0x10^4$cells/well on 12-well plates. The cells were cultured with Dulbecco's modified Eagle's medium (DMEM) (Invitrogen Corporation, Carlsbad, CA) containing 10% fetal bovine serum (FBS) (Invitrogen Corporation), and 1% penicillin/streptomycin (P/S) (Invitrogen Corporation) until further needed.

Effects of ANGII and LOS on C2C12 Myoblasts --- After a 24-hour incubation period, the medium was removed and replaced by low serum-containing medium (DMEM containing 2% horse serum [HS] (Invitrogen Corporation)) containing different concentrations of human ANGII (10^{-10}, 10^{-8}, and 10^{-6}mol/L) or LOS (10^{-10}, 10^{-8}, and 10^{-6}mol/L). The media was replaced with fresh media (containing the same concentrations of ANGII or LOS) every 48 h. All cells were grown for a total of 5 days. To quantify the differentiation of the C2C12 cells, cells were fixed in cold methanol for 2 minutes and washed twice in Dulbecco's phosphate-buffered saline (PBS) for 10 minutes at room temperature (RT). Then incubated in blocking buffer (10% HS in PBS) for 30 minutes at RT. Cells were incubated overnight at 4^oC with mouse monoclonal anti-skeletal fast myosin heavy chain antibodies (clone MY-32, Sigma) in 2% HS in PBS. After washing with PBS, samples were incubated with the secondary antibody (Alexa Fluor 594 donkey anti-mouse IgG, Invitrogen Corporation) in 2% HS for 40 minutes at RT. Cell nuclei were counter-stained with 4',6-diamidino-2-phenylindole dihydrochroride (DAPI, Sigma) for 10 minutes at RT. The fusion index (ratio of nuclei in myotubes to all nuclei) was also determined to evaluate the cell's myogenic differentiation capacity.

In Vivo Evaluation of the Histological and Physiological Effects of LOS on Muscle Healing after Contusion Injury

Animal model --- The policies and procedures followed for the animal experimentation in this study were performed in accordance with those detailed by the US department of Health and Human Services and the National Institutes of Health Guide for the Care and Use of Laboratory Animals. All experimental protocols were approved by the Institutional Animal Care and Use Committee at the University of Pittsburgh (protocol No. 0809009). Ninety normal mice (C57BL/6J, Jackson Laboratory, Bar Harbor and National Cancer Institute at Frederick, Frederick, MD), aged 8-10 weeks weighing 21.4 to 25.8g, were used in this experiment. Six uninjured animals were used as controls to perform physiological analysis to set the normal baseline standard. A muscle contusion injury model was performed on the remaining 82 mice as described previously (5, 18, 34). Briefly, the mice were anesthetized with 1.0 to 1.5% isoflurane (Abbott Laboratories, North Chicago, IL) in 100% O_2 gas. The animal's hindlimb was positioned by extending the knee and plantar flexing the ankle 90 degrees. A 16.2-g, 1.6-cm stainless steel ball (Small Parts Inc, Miami Lakes, FL) was dropped from a height of 100 cm onto an impactor that hit the mouse's tibialis anterior (TA) muscle. The muscle contusion made by this method was a high-energy blunt injury that created a large hematoma which was followed by muscle regeneration (5, 18) similar to that observed in humans following a similar injury (7). The mice were finally sacrificed to evaluate the extent of healing at 1, 2 or 4 weeks after injury.

Drug administration to evaluate the optimal dose of LOS initiated immediately follow injury (dosing study) --- All injured mice were randomly assigned to 1 of 6 groups: (1) no injury and no treatment (uninjured group, n=6); (2) injury with no losartan treatment (control group, n=6); (3) administration of 3mg/kg/day of oral LOS, (Cozaar®, Merck, Whitehouse Station, NJ), initiated immediately after injury (3 mg group, n=6), (4) administration of 10mg/kg/day of oral LOS initiated immediately after injury (10 mg group, n=6), (5) administration of 30mg/kg/day of oral LOS initiated immediately after injury (30 mg group, n=6) (6) administration of 300mg/kg/day of oral LOS initiated immediately after injury (300 mg group, n=6) (Figure 1). The control group was supplied with normal drinking water while the other 4 treatment groups received commercially available LOS diluted in the drinking water. These doses were calculated based on body weight and the average daily intake of fluid *ad libitum* by the mice which was determined by monitoring the mice 1 week before injury. All animals were caged separately and allowed access to the water or LOS solutions *ad libitum* from the time of injury to sacrifice 4 weeks after injury.

Evaluation of the timing administration of a clinically relevant human equivalent LOS dosage (timing study) --- The 10mg/kg/day dose of LOS which is equivalent to the clinical dose used for the treatment of high blood pressure (50mg/day) in humans (38), was used for the timing studies. All injured mice were randomly assigned to 1 of 5 groups: (1) no injury and no treatment (uninjured group, n=6, same as dosing study); (2)

146 injury with no losartan treatment (control group, n=18, included n=6 same as control
147 group in dosing study at 4 weeks after injury); (3) administration of 10mg/kg/day of oral
148 LOS, (Merck, Whitehouse Station, NJ), initiated immediately after injury (day 0 group,
149 n=18, included n=6 same as 10 mg group in dosing study at 4 weeks after injury), (4)
150 administration of 10mg/kg/day of oral LOS initiated at 3 days post-contusion injury (day
151 3 group, n=18), (5) administration of 10mg/kg/day of oral LOS initiated at 7 days post-
152 injury (day 7 group, n=12) (see schematic representation of the in vivo experimental
153 protocol in Figure 1). Losartan was dissolved in drinking water and the dose of 10
154 mg/kg/day was calculated as described above, for the dosing study. All animals were
155 caged separately and allowed access to the water or LOS solutions *ad libitum* from the
156 predetermined initiation time of the LOS treatment (0, 3 or 7 days post injury) to the time
157 of sacrifice 1, 2 or 4 weeks after injury. The expression of MSTN and FSTN were
158 evaluated at 1 and 2 weeks after injury (timing study) and muscle regeneration, fibrosis
159 and strength were evaluated at 4 weeks post-injury (dosing and timing studies).

160 *Physiological evaluation of muscle strength* --- At 4 weeks post-injury,
161 physiological testing was performed on bilateral hindlimbs of all the treatment groups via
162 modified *in situ* force testing as described by Dellorusso, et al. (6). The mice were
163 anesthetized with 1.0–1.5% isoflurane (Abbott Laboratories) and placed on a platform
164 maintained at 37^{o}C using a heating pad. The peroneal nerve was exposed by a lateral
165 incision at the knee which was then hooked to a small electrode. The exposed nerve was
166 kept moist by periodic applications of isotonic saline. To stabilize the hind limb, the knee

and foot were secured to the platform with cloth tape. The foot was tied securely to the lever arm of a 1500A Small Intact Muscle Test System (Aurora Scientific Inc., ON, Canada). The system has Dynamic Muscle Control Software (Aurora Scientific Inc.) to monitor the force of dorsiflexor torque developed by the muscle. All data were displayed and stored on a computer and analyzed with the Dynamic Muscle Analysis Software (Aurora Scientific Inc.).

The anterior crural muscles were stimulated through the peroneal nerve using electrodes. The anterior crural muscles were stimulated with 0.2-ms pulses with the ankle in the neutral position (0^o flexion). It was adjusted for muscle length (L_0) to get the maximum isometric twitch force (peak twitch; P_t). While held at L_0, the muscle was stimulated at increasing frequencies, stepwise from 100Hz by 50Hz, until a maximum force (P_0) was monitored. Then, we performed stimulation three times to measure the length (mean ± SD). A one- to two-minute rest period was allowed between each tetanic contraction. Then, the muscles' length was measured with calipers. Optimum fiber length (L_f) was calculated on the TA, one of the anterior crural muscles. The L_f/L_0 ratio of 0.6 (3). After the evaluation, the mice were sacrificed and the muscles were removed from the leg and weighed prior to being flash frozen in 2-metylbutane pre-cooled with liquid nitrogen. Specimens were kept at -80^oC until sectioning. Total muscle cross-sectional area (CSA) of the TA muscle was calculated by dividing muscle mass by the product of L_f and $1.06mg/mm^3$, the density of mammalian skeletal muscle. Specific P_t and P_0 were calculated by dividing P_t and P_0 by CSA (N/cm^2).

188

189 *Histological evaluation of muscle regeneration and fibrosis formation* --- At 4
190 weeks post-injury, serial cryosections of 8μm thickness were stained with hematoxylin
191 and eosin (H-E) to monitor the number of regenerating myofibers. The total number of
192 centronucleated myofibers, which are the regenerating myofibers (16, 27, 39), within the
193 injury site were quantified using 10 random 200x fields selected from each sample (8-9,
194 31, 34). Results were compared with the control and each of the treatment groups.

195 A Masson's Modified IMEB Trichrome stain Kit (IMEB Inc, Chicago, IL) was
196 used to measure areas of fibrotic tissue within the injury sites. After staining, the ratio of
197 the fibrotic area to the total cross-sectional area of 10 random 100x fields was calculated
198 to estimate fibrosis formation using Northern Eclipse software (Empix Imagining Inc.,
199 Cheekatawaga, NY) in accordance with a previously described protocol (8-9, 31, 34).

200 *Immunofluorescent Staining* --- At 1 and 2 weeks post-injury (timing study),
201 immunohistochemistry was performed on the injured skeletal muscle, to measure the
202 expression of MSTN and FSTN in the control, day 0, day 3 and day 7 groups. The
203 cryosectioned tissue was fixed in 4% formalin for 5 minutes followed by two 10-minute
204 PBS washes. The sections were then blocked with 10% HS for 1 h at room temperature
205 (RT). Sections were incubated overnight at 4°C with primary antibodies (MSTN; gdf8
206 antibody A300-401A, Bethyl Laboratories Inc., Montgomery, TX. FSTN; follistatin sc-
207 23553, Santa Cruz Biotechnology Inc., Santa Cruz, CA) in 2% HS. After washing in PBS,
208 the samples were incubated with secondary antibodies (MSTN; donkey anti-rabbit IgG

conjugated with Alexa Fluor 594, Invitrogen Corporation. FSTN; donkey anti-goat IgG conjugated with Alexa Fluor 488, Invitrogen Corporation) in 2% HS for 1 h at RT. After washing in PBS, counterstaining was performed using DAPI (Sigma-Aldrich, Inc.).

The MSTN or FSTN positive areas (MSTN; red, FSTN; green positive) were calculated using Northern Eclipse software (Empix Imaging Inc). After staining, the total positive area to the total cross-sectional area of 10 randomly selected sections was calculated using a previously described protocol (8-9, 18, 31).

Western Blot analysis --- To determine the levels of MSTN and FSTN in the injured muscle, sodium dodecyl sulfate (SDS)–polyacrylamide gel electrophoresis (PAGE) followed by western blotting was performed using techniques described previously (11-12, 20, 43). Briefly, samples were minced and homogenized in 1 vol. of an isolation buffer (10 mM Tris–HCl, 10 mM NaCl, and 0.1 mM EDTA, pH 7.6). Protein concentration in the homogenates was determined by using a protein assay kit (Bio-Rad, Hercules, CA) and bovine serum albumin (albumin from bovine serum A2153, Sigma) was used as the standard. Each sample was then solubilized in SDS sample buffer: 30%(v/v) glycerol, 5%(v/v) 2-mercaptoethanol, 2.3%(w/v) SDS, 62.5 mM Tris–HCl, 0.05%(w/v) bromophenol blue, at pH 6.8, and 1 mg of protein was loaded on a 12% SDS–polyacrylamide gel. Electrophoresis was carried out at a constant current of 20 mA for 60 min according to the method of Laemmli (21). We investigated the expression of MSTN and FSTN in each of the muscles. Following SDS–PAGE, proteins were

transferred to polyvinylidene difluoride (PVDF) membranes (pore size 0.2 lm, Bio-Rad) using a mini trans-blot cell (Bio-Rad, Inc.) at a constant voltage of 100 V for 60 min at 4 ^{o}C. After the transfer, PVDF membranes were blocked for 1 h using a blocking buffer (3% BSA with 0.1% Tween 20 in Tris-buffered saline (TTBS), pH 7.5) the membranes were incubated overnight at 4^{o}C with a polyclonal antibody (MSTN; gdf8 antibody A300-401A, Bethyl Laboratories Inc., FSTN; sc-23553, Santa Cruz Biotechnology Inc., β-Actin; sc-47778, Santa Cruz Biotechnology Inc.) and then reacted with a secondary antibody (MSTN; goat anti-rabbit immunoglobulin G conjugate to alkaline phosphatase, Sigma-Aldrich, Inc., FSTN; rabbit anti-goat immunoglobulin G conjugate to alkaline phosphatase, Sigma-Aldrich, Inc., β-Actin; Goat polyclonal Secondary Antibody to Mouse IgG - H&L (HRP), ab6789, Abcam Inc., Cambridge, MA) for 2 h. The membranes were subsequently reacted with bromochloroindolyl phosphate–nitroblue tetrazolium substrate. The bands from the immunoblots were quantified using computerized densitometry (SCION Image, Inc.). The expression levels of MSTN and FSTN were divided by the respective β-actin expression levels to normalize total protein content of the samples. Each sample was also investigated in at least triplicate to ensure consistency and obtain a mean with a low standard error. (11-12, 20, 43).

Statistical Analysis---All of the results of this study were expressed as the mean ± SEM. To determine minimum effective dose of LOS *in vivo*, we analyzed the results using Williams' multiple comparison (41, 46). Statistical significance of the data was

analyzed, when necessary, by t-test or ANOVA with Scheffe's post hoc test. Differences were considered significant at $P < 0.05$.

RESULTS:

1 – In Vitro Potential of ANGII and LOS on C2C12 Myoblasts

Effects of ANGII and LOS on Myoblasts --- The fusion index of C2C12 cells in the groups exposed to 10^{-10}, 10^{-8}, and 10^{-6}mol/L of ANGII was significantly lower (0.23±0.06, 0.27±0.08, and 0.23±0.06) than the control group (0.35±0.07) ($p<0.05$, respectively) (Figure 2A-D). On the other hand, groups treated with LOS (10^{-10}, 10^{-8}, and 10^{-6}mol/L) showed higher fusion indices (0.17±0.03, 0.16±0.04, and 0.17±0.05) than the control group (0.12±0.04) ($p<0.05$, respectively) (Figure 2E).

2 – In vivo Evaluation of the Histological and Physiological Effects of Different Doses of LOS on Muscle Healing after Contusion Injury

There was no significant difference in total body weight or in the harvested TA muscle's wet weight at 4 weeks after injury among the 4 groups (3, 10, 30 and 300 mg/kg per day, date not shown).

High Doses of Losartan Improved Muscle Strength Recovery after Contusion Injury --- Results from the physiological evaluations in the dosing study groups performed at 4 weeks post-injury are shown in Figure 3A-B. Immediately after contusion injury, the TA muscles were severely damaged. Two weeks after contusion injury, the specific peak twitch and tetanic forces in the control group were 1.70±0.39 and 4.04±1.70

(10^{-2}N/cm^2), respectively. There was no significant difference among the 4 groups (control, day 0, day 3 and day 7 groups) in this pilot study (data was not shown).

Dosing study --- At 4 week post-injury, the injured control group showed significantly less specific peak twitch and tetanic forces (2.12±0.42 and 7.01±1.32 (10^{-2}N/cm^2)) when compared with the non-injured group (2.89±0.11 and 10.2±2.4 (10^{-2}N/cm^2)). The control group demonstrated improvement in specific peak twitch and tetanic forces compared to the 2week post-injury time point. Specific peak twitch and tetanic forces in the higher LOS dosage groups (30 and 300mg/kg/day; peak twitch 2.75±0.42 and 2.88±0.47 (10^{-2}N/cm^2), and peak tetanic 10.2±2.0 and 10.1±2.0 (10^{-2}N/cm^2)) were greater than the control group; however, in the lower dosage groups (3 and 10mg/kg/day; peak twitch 2.39±0.22 and 2.27±0.34 (10^{-2}N/cm^2), and peak tetanic 7.98±0.99 and 7.92±0.96 (10^{-2}N/cm^2)) did not show an improvement in muscle strength when compared with the control group (Figure 3A-B). The group treated with 300mg/kg/day of LOS showed the best muscle strength recovery for both peak twitch and tetanic forces among the groups. In this study the effective minimum dose of LOS to improve muscle force when injected immediately after injury was 30mg/kg/day.

Timing study --- Specific peak twitch and tetanic forces were significantly greater in the muscles mice that were treated with LOS (10 mg/kg per day) initiated 3 days (3.17±0.34, 10.7±1.6 (10^{-2}N/cm^2)) after injury compared to the control and day 0 groups (peak twitch 2.12±0.42, 2.21±0.58, and peak tetanic 7.48±1.2, 7.92±0.91 (10^{-2}N/cm^2), $p<0.05$ respectively). A similar benefit was also observed when LOS was administered at

292 7 days post-injury (peak twitch 3.00±0.52, peak tetanic 9.70±1.6 (10^{-2}N/cm^2)).
293 Additionally, there was no significant difference between the day 3, day 7 and normal
294 groups. The uninjured group (peak twitch; 2.89±0.11, peak tetanic; 10.2±2.4 (10^{-2}N/cm^2))
295 generated significantly greater specific peak twitch and tetanic forces than the control and
296 day 0 group (Data results above, $p<0.05$ respectively, Figure 4A-B).
297
298 *Effect of LOS on Myofiber Regeneration after Contusion Injury* --- The number of
299 centronucleated regenerating myofibers (16, 27, 39), present in the contusion-injured
300 muscle were counted and compared among the groups 4 weeks after contusion injury in
301 the dosing and timing studies (Figure 3C-G, Figure 4C-F).
302 *In the dosing study*, the group treated with 300mg/kg/day of LOS showed the
303 greatest number of centrally nucleated fibers when compared to the other groups
304 (194.5±68.2/high-powered fields (hpf)) (Figure 3H). The effect of LOS on myofiber
305 regeneration decreased gradually with reductions in the dose of LOS. The minimum
306 effective dose of LOS on muscle regeneration was 30mg/kg/day ($p<0.05$). There were no
307 significant differences between the lower dosage groups (3 and 10mg/kg/day; 108.8±27.8
308 and 94.47±26.6/hpf) and the control group (91.32±23.0/hpf).
309 *In the timing study with 10 mg/kg per day of LOS*, we observed significant
310 increases in the number of centronucleated myofibers in the day 3 and day 7 groups
311 (149.0±25.4, 123.3±28.3/hpf) compared with the other groups (control group;
312 91.32±23.0/hpf and day 0 group; 94.47±26.6/ hpf, $p<0.05$ respectively, Figure 4G).

Effect of LOS on Muscle Fibrosis after Contusion Injury --- The areas of fibrotic scar tissue in the contusion injured muscle were counted and compared among the groups 4 weeks after contusion injury in the dosing and timing study (Figure 3I-M, Figure 4H-K).

In the dosing study, the mean area of fibrosis of the control group within the zone of injury was 12.8±5.0%. The higher LOS dosage groups (30 and 300mg/kg/day) had significantly less fibrosis (9.82±4.9 and 8.48±3.8%) when compared with the control group ($p<0.05$ respectively), while the lower LOS dosage groups (3 and 10mg/kg/day; 12.4±4.6 and 13.0±7.5%) did not show any significant difference compared with the untreated control group (Figure 3N).

In the timing study with 10 mg/kg per day of LOS, the day 3 and day 7 groups showed significantly less area of fibrosis (1.87±1.0% and 4.17±3.1%, respectively) compared with the control and day 0 groups (12.8±5.0%, 12.4±4.6%, $p<0.05$ respectively, Figure 4L).

LOS enhanced expression of Follistatin and Myostatin in the timing study --- Immunohistochemical staining was performed to detect FSTN and MSTN expression in the contusion injured TA muscles 1 and 2 weeks after injury (FSTN; green area, MSTN; red area, Figure 5A-C, Figure 6A-D). The 1week time point only contains data from 3 groups (control, day 0 and day 3), due to the fact that LOS treatment was just initiated for the day 7 group (i.e. 1 week post-injury). The MSTN-positive areas were measured and compared between the various treatment groups. Expression of MSTN in the day 3 group

(8.80±5.5%) was significantly lower than in the control and day 0 groups (28.1±20.1%, 24.9±14.2%) at 1 week after injury ($p<0.05$ respectively, Figure 5D). Two weeks after injury, there was no significant difference between the groups (control; 13.6±7.5%, day 0; 12.5±6.5%, day 3; 9.79±6.0%, day 7; 11.1±8.5%, Figure 6E). The FSTN-positive areas were measured and compared between the various treatment groups. Expression of FSTN in the day 3 group (3.98±1.2%) was greater than the control and day 0 groups (1.04±0.56%, 1.20±0.46%) at 1 week after injury ($p<0.05$ respectively, Figure 5E). The day 3 and day 7 groups (3.70±1.9%, 2.70±1.3%) were significantly greater than the control and day 0 groups (0.82±0.6%, 1.22±0.56%) 2 weeks after injury ($p<0.05$ respectively, Figure 6F).

These immunohistochemical findings were further confirmed using western blot analysis. Expression of MSTN in the day 3 group (1.61±0.5 Arbitrary OD units) was significantly lower than in the untreated control and day 0 groups (4.14±1.2, 3.36±1.5 Arbitrary OD units) at 1 week after injury ($p<0.05$, Figure 5F, H). However, two weeks after injury, there was no significant difference between the groups (control; 1.77±0.2, day 0; 1.66±0.4, day 3; 1.52±0.3, day 7; 1.62±0.4 Arbitrary OD units) (Figure 6G, I). Expression of FSTN in the day 3 group (4.09±1.2 Arbitrary OD units) was significantly greater than in the control and day 0 groups (1.97±1.0, 2.04±1.0 Arbitrary OD units) 1 week after injury ($p<0.05$, Figure 5G, I). The day 3 and day 7 groups (3.54±1.2, 2.88±1.3 Arbitrary OD units) were also significantly greater than the control and day 0 groups (1.31±0.5, 1.28±0.7 Arbitrary OD units) 2 weeks after injury ($p<0.05$, Figure 6H, J).

DISCUSSION:

The functional recovery of skeletal muscle injury is the most important parameter for the clinical translation of this therapy. Our results demonstrated that LOS accelerated the healing of injured muscle when administered immediately following injury; however, the minimum dose of LOS used to obtain these results was 30mg/kg/day, which is 3 times the dose recommended for use in human patients suffering from hypertension. Additionally, this study dramatically demonstrated that the timing of LOS administration is critical for obtaining optimal results when using a clinically relevant safe human dose of LOS (10mg/kg/day initiated 3 and 7 days post-injury).

The role of ANGII in muscle fibrosis formation after injury is well documented in the cardiac literature, in which the antagonism of ANGII with ARBs is noted to significantly improve cardiac contractility and cardiac output (13). We demonstrated that LOS could enhance muscle healing by reducing fibrosis and enhancing muscle regeneration (2). It was previously reported that LOS diminished angiotensin II–induced type I collagen over-expression via the TGF-β signaling pathways which is mediated by the angiotensin II type 1 receptor (AT1) (42). We have also reported that the expression of AT1 was elevated in the zone of injury and was more densely distributed within the extracellular matrix induced fibrotic tissue which indicates that AT1 up-regulation is intimately related to the deposition of fibrous scar tissue (2). Furthermore, it has

375 previously been reported that ANGII protects endothelial cells against apoptosis and high
376 doses of LOS are able to completely block this anti-apoptotic effect (32).
377 Moreover, ANGII plays a critical role in inducing proliferation, differentiation and
378 inflammation after injury in skeletal muscle via AT1 through the release of mitogen-
379 activating protein kinases (MAPK) (17, 30, 36). that the effect of high doses of LOS
380 administrated immediately after injury may reduce the apoptotic effect of ANGII and
381 decreases the deposition of fibrous scar tissue after injury.
382 Skeletal muscle development and regeneration are highly organized processes that
383 are under tightly balanced regulation. MSTN, which is a member of the TGF-β
384 superfamily, is expressed in adult skeletal muscle, heart and adipose tissue (40) and acts
385 mainly as a negative regulator of tissue growth (47). MSTN is expressed during
386 embryogenesis and in adult skeletal muscles during regeneration. High levels of MSTN
387 are detected within necrotic fibers and connective tissue during the degenerative phase of
388 muscle repair. On the contrary, the regenerating myotubes contained a low level of
389 MSTN during enlargement and fusion (19). This expression profile suggests that MSTN
390 acts as an inhibitor of muscle growth, perhaps via the repression of satellite cell
391 proliferation during the process of muscle regeneration. It has been shown that MSTN
392 inhibits cell proliferation and protein synthesis in C2C12 muscle cells (44), including the
393 expression of MyoD and Pax3 (22).
394 Administration of LOS 3 and 7 days after contusion injury, using the clinically
395 relevant human dose equivalent for treating of hypertension (10mg/kg/day), effectively

leads to the down regulation of endogenous MSTN at 1 week post-injury. We also observed an increase in the number of centronucleated myofibers, a decrease in the area of fibrosis, and an enhancement in muscle strength when LOS was administered 3 days after muscle injury. Wang et al. have demonstrated that LOS significantly blocked the increase of the MSTN protein by blocking ANGII, which indicates that ANGII increases MSTN expression through the AT1 receptor (45). These results suggest that LOS administrated 3 and 7 days after injury can diminish ANGII–induced MSTN deposition of type I collagen which is mediated by the AT1 signaling pathway which would then allow for accelerated muscle cell proliferation and regeneration.

In addition, the expression of FSTN was observed to be up-regulated in the day 3 and 7 groups up to 2 weeks after injury. It is well known that FSTN, a secreted protein, is able to bind and neutralize the actions of many members of the TGF-β superfamily of proteins. FSTN was first implicated in the regulation of follicle-stimulating hormone secretion in the pituitary and subsequently in other regions of the adult body associated with reproductive function (35). In skeletal muscle, FSTN stimulates satellite cell proliferation (10) and FSTN gene therapy has shown promise as a MSTN inhibitor to treat the diseased muscles of mdx mice (a mouse model of Duchenne muscular dystrophy) by increasing muscle size and strength and reducing fibrosis (37). Our results support these latter findings, since injured muscle treated with LOS shows an increase in FSTN expression, a decrease in MSTN expression, an increase in muscle regeneration and a decrease in fibrosis formation. It remains unclear if LOS treatment acts directly

through the enhancement of FSTN and inhibition of MSTN, or if the mechanism is completely via the up-regulation of FSTN which then inhibits the action of MSTN, or a combination of both.

The mechanism which was responsible for the dramatic differences observed in muscle healing between the day 3 and 7 treatment group and the other groups is the most compelling question about the current study, i.e. why does initiating the administration of 10mg/kg/day of LOS at day 0 verses day 3 verses day 7 post- injury have such a dramatic effect on the expression of FSTN in the skeletal muscle signaling pathways, with the day 3 group being significantly better than the other treatment groups. In the current study, we considered the stages associated with the healing process of injured skeletal muscle to determine the timing of LOS administration. The process of muscle healing includes degeneration and inflammation, which overlap in their timing, followed by muscle regeneration and finally with fibrosis deposition at the injury site (15); therefore, we decided to administer LOS on day 0 (preinflammation), day 3 (early post–inflammation) and day 7, which marks the beginning of the fibrosis stage. We posit that the regulation of MSTN is very important for muscle healing in the early stages of the process for proper skeletal muscle healing; hence the timing of administering LOS, using a safe human dose, is important for properly regulating MSTN expression after injury. We also suggest that there are some healing signals during the inflammation stage after muscle injury such as MAPK expression, (17, 30, 36) so administering LOS immediately after injury would interfere with this signaling pathway that is involved in the healing process;

however, administering LOS after the inflammation stage at 3 and 7 days post-injury would not interfere with this signaling process but would reduce MSTN expression later in the process and inhibit fibrosis formation. Additional investigation is essential to determine the exact mechanisms involved in the skeletal muscle healing process induce via LOS muscle regenerative therapy.

This is one of the limitations of this type of testing approach, we could not completely separate the force generated by the TA from the EDL; however, it is more clinically relevant to measure the function of dorsi ankle flexion than only measuring the length of the TA muscle.

In summary, we have demonstrated that the administration of a high dose (30 and 300mg/kg/day) of LOS immediately after injury could accelerate muscle healing, but not when the recommended human equivalent dose used for the treatment of hypertension of 10mg/kg/day was administered immediately following injury. Interestingly, administering 10mg/kg/day of LOS 3 and 7 days after skeletal muscle contusion injury could significantly enhance the structural and functional healing of skeletal muscle in mice. This study demonstrated that LOS used at the safe dosage concentration used to treat hypertension in humans can be mediated directly through the inhibition of MSTN and/or the up-regulation of FSTN, which then inhibits MSTN or through a combination of both. This study strongly suggests that the use of LOS to expedite the muscle healing process could also be applied to other types of acute muscle injuries, including severe

458 limb threatening conditions such as compartment syndrome, and may also be useful for
459 the treatment of degenerative fibrotic muscle disorders.
460
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592 FIGURE LEGENDS:

593 Figure 1. Schematic representation of the in vivo experimental design. Dosing study;
594 uninjured, no injury without LOS treatment (n=6); control group, Injured without LOS
595 treatment (n=6); 3mg group, 3mg/kg/day of LOS administrated immediately after injury
596 (n=6); 10mg group, 10mg/kg/day of LOS administrated immediately after injury (n=6);
597 30mg group, 30mg/kg/day of LOS administrated immediately after injury (n=6); 300mg
598 group, 300mg/kg/day of LOS administrated immediately after injury (n=6).
599 Timing study; uninjured, no injury without LOS treatment (n=6); control group, injured
600 without LOS treatment (n=18); day 0 group, 10mg/kg/day of LOS administrated
601 immediately after injury (n=18); day 3 group, 10mg/kg/day of LOS administrated 3 days
602 after injury (n=18); day 7 group, 10mg/kg/day of LOS administrated 7 days after injury
603 (n=12). All animals in the dosing study were sacrificed 4 weeks after injury (n=6,
604 respectively). Six animals in each group of the timing study were sacrificed 1, 2 or 4
605 weeks after injury. The date and specimen of the uninjured and control groups in the
606 dosing study were the same as the timing study at 4 weeks after injury. Also, the date and
607 specimen of the 10mg/kg/day group in the dosing study was same as day 0 group at 4
608 weeks after injury.
609

610 Figure 2. Immunocytochemical staining of C2C12 cells for fast myosin heavy chain 5
611 days after incubation with different concentration of ANGII or LOS (A, control; B, 10^{-8}
612 mol/L of angiotensin II; C, 10^{-8}mol/L of LOS). Myotubes are shown in red and nuclei

613 are in blue (original magnification, ×200). D and E, comparison of fusion index of
614 C2C12 differentiation with varied concentrations of ANGII or LOS, respectively. *;
615 P<0.05 vs control by ANOVA.
616
617 Figure 3. LOS enhanced muscle force in a dose dependent manner. Comparisons of
618 specific peak twitch (A) and tetanic (B) force (10^{-2}N/cm^2). *; *P<0.05* vs control, 3 and
619 10mg/kg/day groups by ANOVA. Histologic evaluation of regenerating myofibers and
620 the formation of scar tissue at 4 weeks after contusion injury by H-E staining (C-G) or
621 Masson's trichrome staining (I-M) (collagen: blue, myofibers: red, nuclei: black) of TA
622 muscles treated with different concentration of LOS (C and I, control; D and J,
623 3mg/kg/day; E and K, 10mg/kg/day; F and L, 30mg/kg/day; and G and M,
624 300mg/kg/day) initiated immediately after injury. Regenerating myofibers were defined
625 by centronucleated myofibers (original magnification, ×200). The graph (H) depicts
626 quantification in the number of regenerating myofibers in LOS treated animals compared
627 with control animals. *; P<0.05 vs control by ANOVA. The ratio of the fibrotic area to
628 the total cross-sectional area of 10 randomly selected slices was calculated to estimate the
629 fibrosis formation (original magnification, ×100). The graph (N) depicts quantification of
630 fibrotic area in LOS treated animals compared with control animals. Values are given as
631 the means ± SEM. n=6 per group. hpf; high-power field. *; P<0.05 vs control by
632 ANOVA.
633

Figure 4. LOS (10 mg/kg per day) administration enhanced muscle force in a time dependent manner. Comparisons of specific peak twitch (A) and tetanic (B) force (10^{-2}N/cm^2). Values are given as the means ± SEM. n=6 per group. *; *P<0.05* vs control and day 0, **; *P<0.05* vs control, by ANOVA. Histological evaluation of regenerating myofibers and the formation of scar tissue at 4 weeks after contusion injury by H-E staining (C-F) or Masson's trichrome staining (H-K) (collagen: blue, myofibers: red, nuclei: black) of TA muscle treated with different timing of LOS administration (C and H, control; D and I, day 0; E and J, day 3; F and K, day 7) initiated after injury. Regenerating myofibers were defined by centronucleated myofibers (original magnification, ×200). The graph (G) depicts quantification in the number of regenerating myofibers in LOS treated animals compared with control animals. *; P<0.05 vs control and day 0 by ANOVA. The ratio of the fibrotic area to the total cross-sectional area of 10 randomly selected slices was calculated to estimate the fibrosis formation (original magnification, ×100). The graph (L) depicts quantification of fibrotic area in LOS treated animals compared with control animals. Values are given as the means ± SEM. n=6 per group. hpf; high-power field. *; P<0.05 vs control and day 0 by ANOVA. Abbreviations are the same as depicted in Figure 1.

Figure 5. LOS effect on MSTN and FSTN expression in the injured skeletal muscle, 1 week after injury. Immunohistochemical evaluation of MSTN and FSTN expression by immunofluorescence staining of the TA muscles. The sections were immunostained with

MSTN (red), FSTN (green) and cell nuclei (blue) (A, control; B, day 0; C, day 3). The ratio of the MSTN or FSTN positive area to the total cross-sectional area of 10 randomly selected slices was calculated. The graph depicts quantification of the MSTN (D) or FSTN (E) positive areas in LOS treated animals compared with each group. LOS reduced the expression of MSTN (F, H) and enhanced FSTN expression (G, I) when measured by western blotting. Values are the means ± SEM. n=6 per group. *; $P<0.05$ vs control and day 0 by ANOVA. Abbreviations are the same as in Figure 1.

Figure 6. LOS effect on MSTN and FSTN expression at 2 weeks after injury. Immunohistochemical evaluation of the expression of MSTN and FSTN by immunofluorescent staining of the TA muscles. The sections were immunostained with MSTN (red), FSTN (green) and cell nuclei (blue) (A, control; B, day 0; C, day 3; D, day 7). The ratio of the MSTN or FSTN positive area to the total cross-sectional area of 10 randomly selected slices was calculated. The graph depicts quantification of the MSTN (E) or FSTN (F) positive area in LOS treated animals compared with each groups. LOS reduced the expression of MSTN (G, I) and increased FSTN expression (H, J) when measured by western blot analysis. Values are the means ± SEM. n=6 per group. *; $P<0.05$ vs control and day 0 by ANOVA. Abbreviations are the same as in Figure 1.

Combination Treatment of Platelet-Rich Plasma and Angiotensin II Receptor Blocker for Contusion Skeletal Muscle Injury in Mice

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INTRODUCTION:

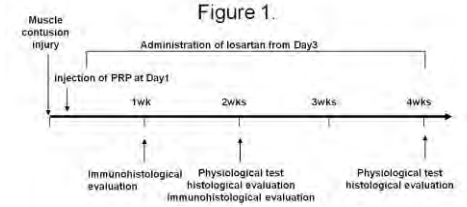
Although muscle contusions are capable of healing, incomplete functional recovery often occurs. We have previously reported that when a safe human dose of losartan (10mg/kg/day), one of the FDA approved Angiotensin II Receptor Blockers (ARBs) and anti-fibrotic agent, that block TGF-β1, was administrated 3 days after injury it can promote functional improvement, muscle regeneration and decrease fibrosis at 4 weeks after injury [1]. Moreover, some reports have shown that Platelet-Rich Plasma (PRP), which includes many kinds of growth factors, including TGF-β1, can accelerate muscle healing after injury [2]. Our hypothesis that losartan treatment along with PRP can further accelerate the muscle healing process compared to the use of losartan or PRP treatment alone.

The purpose of the current study is to investigate the potential functional improvement of contusion injured skeletal muscle in mice using both losartan and PRP in combination.

METHODS:

The contusion injury was created on the tibialis anterior (TA) muscle of C57BL/6 wild-type mice. All injured mice were randomly assigned to 1 of 4groups: (1) fed plain drinking water (control group, n=14); (2) administered 10mg/kg/day of oral losartan starting 3 days (losartan group, n=14), (3) injected with 20 μl of PRP in the injured TA muscle 1 day after injury (PRP group, n=14), (4) combined treatment as (2) and (3) (PRP/losartan group, n=14). PRP was isolated from the rat whole blood via a double centrifuge technique. The concentration of the platelets obtained in the PRP was 5.5 times higher than that of the whole blood. All animals were sacrificed at 1, 2 and 4 weeks post-injury to evaluate, histologically and physiologically, muscle healing (Fig.1).

Figure 1.



Histological evaluation was performed using hematoxlin and eosin staining to monitor the number of regenerating myofibers, and Masson's trichrome staining was used to measure areas of fibrotic tissue within the injury sites. Immunohistochemistry was performed to evaluate angiogenesis in the injured site. Statistical analysis was performed with Scheffe's F test as a post hoc test. Statistical significance was defined as $p < 0.05$.

RESULTS:

Injection of PRP enhanced muscle regeneration in injured muscle:

After hematoxlin and eosin staining, the centronucleated regenerating myofibers in the injured muscle were counted and compared among the groups at 2 weeks post-injury. PRP treated groups showed significantly higher numbers of regenerating myofibers (PRP group, 124.9±20.7; PRP/losartan groups, 140.4±19.1/hpf) compared with control group at 2 weeks post-injury (54.9±14.5 /hpf) (Fig. 2A).

Administration of losartan decreased fibrosis formation in injured muscle:

After Masson's trichrome staining, the area of fibrotic scar tissue was evaluated and compared among the groups at 4 weeks post-injury. Losartan treated groups showed significantly less fibrotic area ((losartan group; 4.05±2.35% and PRP/losartan group; 2.05±1.30%) compared with control and PRP groups (21.42±6.14 and 9.78±3.40%, respectively) (Fig. 2B).

Injection of PRP and the administration of losartan showed rapid and greater improvement of muscle strength:

At 2 weeks post-injury, PRP treated groups (PRP and PRP/losartan groups) showed significantly greater specific peak twitch and tetanic forces (twitch; 42.5±12.5 and 46.5±10.2, tetanic; 77.9±11.5 and 87.0±26.5g/cm^2) than the contol group (twitch; 24.1±8.9, tetanic; 47.6±7.26 and g/cm^2, respectively).

Interestingly, PRP/losartan group showed significantly greater specific peak twitch and tetanic forces (twitch; 60.2±11.2, tetanic; 112.2±25.9g/cm^2, respectively) than the other groups at 4 weeks after the injury (control group: twitch; 24.4±5.1, tetanic; 59.1±13.3, losartan group: twitch; 39.2±3.0, tetanic; 81.9±23.2, and PRP group: 39.9±13.8, tetanic; 84.2±20.5g/cm^2, respectively). Moreover, there was no significant difference between the PRP/losartan group and non-injected group (twitch; 59.5±18.7, tetanic; 125.4±30.4g/cm^2, respectively) (Fig. 3).

Injection of PRP and the administration of losartan enhanced angiogenesis and reduced p-Smad2/3 in injured muscle:

CD31expressing areas in the injured TA muscles were measured at 1week post-injury.The PRP injected groups (PRP, PRP/losartan groups) showed significantly greater angiogenesis areas (4.17±1% and 5.18±1.61%, respectively) when compared with control group and losartan group (0.94±0.82 and 1.38±1.29 %, respectively) (Fig. 4A).

pSmad2/3 areas in the injured TA muscles measured at 2 weeks post-injury. Losartan treated groups (losartan group; 0.82±1.15%, PRP/losartan group; 0.92±0.54%) showed significantly less pSmad2/3 positive area than control and PRP groups (4.22±1.57 % and 3.22±0.59 %, respectively) (Fig. 4B).

Figure 2

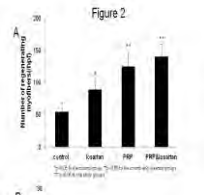


Figure 3.

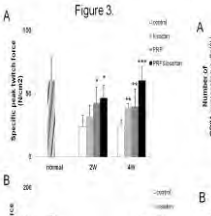
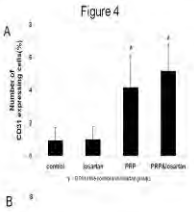


Figure 4



DISCUSSION:

The combination treatment using losartan and PRP following a contusion injury can accelerate skeletal muscle healing. We observed a larger number of regenerating myofibers, greater angiogenesis, less fibrosis, and better functional recovery in the PRP/losartan group. These results suggest that the combination treatment of PRP and losartan after skeletal muscle injury could be more effective than the individual treatments alone and the beneficial effect of combining PRP and losartan is likely related to the inactivation of TGF-β1 within the PRP through losartan treatment.

Significance:

Our study will be helpful to contribute to the development of biological treatments to accelerate muscle healing.

ACKNOWLEDGEMENTS:

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The use of an Anti-Fibrotic Agent Improves the Effect of Platlet Rich Plasma on Muscle Healing after Injury

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ABSTRACT

Background: Muscle contusions are common muscle injuries frequently encountered in atheletes and military personnel. Although these injuries are capable of healing, incomplete functional recovery often occurs through the development of fibrosis.

Hypothesis: The combinatorial use of both platelet-rich plasma (PRP) injection and oral administration of losartan (an anti-fibrotic agent) can enhance muscle healing by stimulating muscle regeneration and angiogenesis and preventing fibrosis in contusion injured skeletal muscle.

Study Design: Controlled laboratory study

Methods: Twenty microliters of PRP was injected into the contusion injured tibialis anterior muscles of mice 1 day post-injury followed by the administration of 10mg/kg/day of losartan 3 days post-injury and continued until the endpoint of the experiment. Muscle regeneration and fibrosis formation were evaluated by histological analysis and functional recovery was measured by physiological testing.

Results: Combined PRP/losartan treatment significantly promoted muscle regeneration, angiogenesis, and muscle function when compared to the other groups. Combined PRP/losartan treatment significantly decreased fibrosis formation and pSmad 2/3 expression when compared to the PRP treatment group and increased vascular endothelial growth factor (VEGF) expression when compared to the losartan treatment group. Furthermore, follistatin (FLST) expression, a positive regulator of muscle growth, was found to be expressed at high levels in the PRP/losartan group when compared to the other groups.

Conclusion: PRP/losartan combinatorial therapy improved overall skeletal muscle healing after muscle contusion injury by enhancing angiogensis and FLST expression and by reducing the expression of pSmad2/3 and hence the formation of fibrosis. These results suggest that PRP therapy after muscle contusion requires TGF-β1 blockade to improve muscle healing.

Clinical Relevance: These findings could contribute to the development of biological treatments to aid in muscle healing after skeletal muscle injury.

Keywords: Skeletal muscle contusion injury; platelet-rich plasma; Angiotensin II receptor blocker; fibrosis; angiogenesis; follistatin

Study Design: Controlled Laboratory Study

INTRODUCTION

Platelet-rich plasma (PRP) is an autologous blood-derived product that possesses an increased concentration of growth factors and secretory proteins [2,19,23]. It has been reported that PRP can influence myoblast, proliferation[46], fibroblast proliferation and migration[3] *in vitro*. In addition, several studies have reported that PRP can enhance angiogenesis because of the numerous growth factors it contains [7 28].Therefore, many research teams have investigated the potential use of PRP to treat pathological conditions in articular cartilage, tendon, ligament, bone, and skeletal muscle [19 34 35]. Although for tendon and ligament injury, an increase of fibrosis formation can accelerate the healing process [6 24], the presence of high concentrations of transforming growth factor beta 1 (TGF-β1) in the PRP could potentially promote fibrosis in injured skeletal muscle.

Increases in fibrosis formation after muscle injury contributes to the risk of re-injury [47]. To obtain better healing after muscle injury, we have previously demonstrated that the use of antifibrotic agents such as decorin[21], relaxin[36], gamma interferon[20], and suramin[38], can enhance muscle regeneration and inhibit fibrosis by blocking TGF-β1 after skeletal muscle injury. Unfortunately, some of these compounds can not readily be translated to clinical practice due to the lack of an oral formulation, Food and Drug Administration (FDA) approval, and/or a relatively severe side-effect profile.

Losartan was the first orally active, commercially available, non-peptide angiotensin II type 1 receptor (AT1) blocker and is a well known antihypertensive drug. It is widely used for the treatment of hypertension and congestive heart failure [4]. Its anti-TGF-β1 effect has been shown to be clinically relevant in many fibrotic disease states such as renal disease, pulmonary fibrosis, cardiomyopathy and aortic aneurysm [1,17,32, 48]. In skeletal muscle, it has been reported that TGF-β1 has a negative effect on satellite cell differentiation during the muscle regeneration process [25]. It has also been reported that blocking TGF-β1 with losartan can increase myoblast proliferation and fusion *in vitro* [18]. Chon et al reported that losartan improved muscle morphology, reduced TGF-β1 signaling and enhanced muscle regeneration in a mouse model of Marfan syndrome [12 ;

hence, losartan's ability to enhance muscle regeneration appears to be due, at least in part, to its ability to inhibit TGF-β1 [5, 12]. Recently we and others reported that follistatin (FLST), a myostatin binding protein, can inhibit myostatin activity and promote muscle growth through the blockade of the pSmad2/3 signaling pathway [31 52]. Moreover, our research team has previously demonstrated that the systemic administration of losartan could reduce muscle fibrosis, increase the number of regenerating myofibers, and enhance the physiological functional of injured muscle [5]. Furthermore, our group has shown that the oral administration of 10mg/kg/day of losartan, which is the recommended safe human dose for the treatment of hypertension, 3 or 7 days post-injury can dramatically improve skeletal muscle healing following contusion injury (unpublished data, 2011).

In the current study, we hypothesized that combining PRP and losartan treatments could substantially improve skeletal muscle healing after contusion injury more effectively than PRP or losartan treatment alone.

MATERIALS AND METHODS

Isolation of Platelet-rich Plasma

Whole blood was collected from adult wild type mice (C57BL/6J, Jackson Laboratory, Bar Harbor and National Cancer Institute, Frederick, MD) via cardiac puncture and transferred into tubes containing 3.2% sodium citrate. PRP was then separated from the whole blood by centrifugation. PRP was isolated from the whole blood via a double centrifugation technique that has been previously described by Plachokova AS, et al [42]. Briefly, the blood sample was centrifuged at 160 g for 20 min at 22°C to separate the plasma containing the platelets from the red cells. The plasma was drawn off the top and centrifuged again at 22°C for an additional 15 min at 400 g to separate the platelets from the plasma. The platelet-poor plasma (PPP) was then drawn off the top, leaving the PRP. The platelet concentration of the PRP was then determined

using a previously described method recommended by the International Committee for Standardization in Hematology [8].

Measurement of TGF -β1 Concentration in the Isolated PRP

A quantitative assay using an enzyme linked immunosorbent assay (mouse TGF- β1 DuoSet ELISA Development System, R&D systems, Minneapolis, MN) was performed to assess the concentration of TGF-β1 in the newly isolated PRP. The PRP and PPP were stored at -80°C and thawed just prior to use. The manufacturer's protocol was followed to determine the concentration of TGF-β1 in the PRP and PPP.

Animal Model

The policies and procedures followed for the animal experimentation performed in this study are in accordance with those detailed by the U.S. Department of Health and Human Services and the National Institutes of Health *Guide for the Care and Use of Laboratory Animals*. All experimental protocols were approved by the Unviversity of Pittsburgh's Institional Animal Care and Use Committee (IACUC # 1005846A).

Seventy-two wild type (WT) mice (C57BL/6J, Jackson Laboratory), aged 8-10 weeks and weighing between 23.4 to 28.2 g, were used in this experiment. A muscle contusion injury was created in the mice as described previously [13 27 38]. Briefly, the mice were anesthetized with 1.0 to 1.5% isoflurane (Abbott Laboratories, North Chicago, Illinois) in 100% O_2 gas and the animal's hindlimb was positioned by extending the knee and plantar flexing the ankle to 90 degrees. A 16.2g/1.6cm stainless steel ball (Small Parts Inc, Miami Lakes, FL) was dropped from a height of 100 cm onto an impactor that reached the mouse's tibialis anterior (TA) muscle. The muscle contusion created is a high-energy blunt injury that results in a large hematoma which is followed by massive muscle regeneration, simulating the healing process seen in humans [16]. All animals were sacrificed at 1, 2 and 4 weeks post-injury to evaluate the muscle healing process,

histologically and physiologically. Three mice (6 muscles) per group were assessed histologically at 1, 2 and 4 weeks post-injury, four mice (8 muscles) per group were assessed physiologically before sacrifice at 2 and 4 weeks post-injury, and four mice (8 muscles) were used as normal controls for the physiological testing.

Administration of Losartan and Injection of PRP

All injured mice were randomly assigned to 1 of 4 groups. Control and PRP groups (n=17 mice/each group) were supplied with normal drinking water from day 1 post-injury until the endpoint of the experiment. Losartan and PRP/losartan groups (n=17 mice/group) received commercially available losartan (Cozaar®, Merck) dissolved in their drinking water at the dosage level of 10mg/kg/day, which is equivalent to the clinical dose given to treat human patients with high blood pressure (50mg/day) [40], from day 3 post-injury until the experimental endpoint (Fig. 1). These doses were calculated based on the average body weight of the mice and their daily fluid intake, which had been monitored for one week prior to injury. All animals were caged separately and allowed access to water or the losartan solution *ad libitum* from the time of injury to sacrifice. At 1 day post-injury, 20ul of PRP was injected directly into the contusion injured region, as previously described [44 45]. The investigators were blinded to the solution contents injected into each group of the mice, i.e. phosphate-buffered saline (PBS) only (control and losartan group, n=17 mice/group) or PRP (PRP and Losartan/PRP groups, n=17 mice/each group) (Fig. 1).

Histological Evaluation of Muscle Regeneration and Fibrosis Formation after Losartan and/or PRP Therapy

Isolated TA muscles were flash frozen in 2-metylbutane pre-cooled with liquid nitrogen at 1, 2, and 4 weeks post-injury. Frozen specimens were kept at -80°C until sectioning. Serial cryosections of 8μm thickness were stained with hematoxylin and eosin (HE) to evaluate the number of regenerating myofibers within the injury sites at 2 weeks post-injury. The total number

of centronucleated myofibers, which are known to be the regenerating myofibers [26] , within the injury site were quantified by selecting 10 random 200x high-powered fields (hpf) selected from each sample [21 20 33 38 41]. Results from the control and each of the treatment groups were then compared.

A Masson's Modified IMEB Trichrome Stain Kit (IMEB Inc, Chicago, Illinois) was used to measure areas of fibrotic tissue within the injury sites at 4 weeks post-injury. After staining, the ratio of the fibrotic area to the total cross-sectional area of 10 randomly selected sections was calculated to estimate the percentage of fibrosis formation using Northern Eclipse software (Empix Imagine Inc., Cheekatawaga, NY) in accordance with a previously described protocol [34 42]. The results were then compared among the different groups.

Physiological Evaluation of Muscle Strength Recovery after Losartan and/or PRP Therapy

At 2 and 4 weeks post-injury, physiological testing was performed using a modified in situ force test as described by Dellorusso, et al [15]. The mice were anesthetized with 1.0–1.5% isoflurane (Abbott Laboratories) and placed on a platform and maintained at 37^{o}C using a heating pad. The peroneal nerve was exposed by a lateral incision at the knee and was hooked to a small electrode. The exposed nerve was kept moist by periodic applications of isotonic saline. To stabilize the hindlimb, the knee and foot was secured to the platform with cloth tape and the foot was tied securely to the lever arm of the 1500A Small Intact Muscle Test System (Aurora Scientific Inc., Ontario, Canada). The system utilizes Dynamic Muscle Control Software (Aurora Scientific Inc.) to monitor the force developed by the muscle. All data were displayed and stored on a computer and analyzed using the Dynamic Muscle Analysis Software (Aurora Scientific Inc.).

The anterior crural muscles were stimulated via the peroneal nerve with the attached electrodes. The TA muscle was stimulated with 0.2-ms pulses. Stimulation voltage and subsequently muscle length (L_0) were adjusted for maximum isometric twitch force (peak twitch;

P_t). While held at L_0, the muscle was stimulated at increasing frequencies, stepwise from 100Hz by 50Hz increments, until the maximum force (P_0) was monitored. A one- to two-minute rest period was allowed between each tetanic contraction and the muscle length was then measured with calipers. Optimum fiber length (L_f) was calculated by the TA L_f/L_o ratio of 0.6 [10]. After the evaluation, the muscle was removed from the leg, the mice were euthanized, and the muscle was weighed. Total muscle cross-sectional area (CSA) of the TA muscles were calculated by dividing muscle mass by the product of Lf and 1.06 mg/mm (the density of skeletal muscle). Specific P_t and P_0 were calculated by dividing P_t and P_0 by CSA (g/cm^2).

Physiological analysis was also performed in three normal mice as a non-injured normal control group.

Immunofluorescent Staining

At 1week post-injury, immunohistochemistry using antibodies against VEGF and CD31 was performed to detect their expression at the injury site using protocols previously described (See table 1). Also at 2 weeks post-injury, pSmad2/3 and follistatin expression was detected via immunohistochemistry. Expression levels were quantified using Northern Eclipse software (Empix Imaging Inc.) using a previously described protocol. [27 30 38 41].

Statistical Analysis

All of the results from this study were expressed as the mean±standard deviation. All histological results were statistically analyzed using one-way ANOVA performed with SPSS software (IBM, Inc., Armonk, New York), with a subsequent Scheffe post hoc analysis. The differences in specific twitch and tetanic force measured during the physiological testing were analyzed using the unpaired t-test among each group. Statistical significance was defined as $P < 0.05$.

RESULTS

Platelet Count and TGF-β1 Concentration

The platelets in the PRP used in these studies exhibited normal morphology. Platelet counts revealed that the PRP preparation technique produced a highly concentrated platelet containing plasma. The average whole blood platelet count was 38.0±4.5 x 10^4 platelets/ml, whereas the average PRP platelet count was 208.0±25.8 x 10^4 platelets/ml, which is a 5.5 fold increase in the number of platlets.

The concentration of TGF− β 1 in the freshly isolated PRP was 16.4 times higher than that found in the PPP. (At a 1:60 dilution the PRP was found to have 66,474.7 pg TGF–β1/ml while the PPP had only 4,034.2 pg TGF–β1/ml)

PRP Treatment Accelerated Muscle Regeneration in Injured Muscle

The centronucleated regenerating myofibers in the injured muscles were counted and compared among the groups at 2 weeks post-injury. (Fig.2A) At 2 weeks post-injury, the PRP treated groups (PRP and PRP/losartan groups) contained numerous regenerating myofibers at the injury site. PRP treated groups showed significantly higher numbers of regenerating myofibers (PRP group, 124.9±20.7; PRP/losartan groups, 140.4±19.1/hpf) compared with the control group at 2 weeks post-injury (54.9±14.5 /hpf). No differences were observed between the PRP and the PRP/losartan groups. (Fig.2B)

Treatment with Losartan and PRP Reduced the Formation of Muscle Fibrosis.

After Masson's trichrome staining, the area of fibrotic scar tissue was evaluated and compared among the groups at 4 weeks post-injury. (Fig.3A) The control group showed significantly larger fibrotic areas than the other groups. The losartan treated groups showed significantly less fibrotic area (losartan group; 4.05±2.35% and PRP/losartan group; 2.05±1.30%)

compared with the control and PRP groups (21.42±6.14% and 9.78±3.40%, respectively). (Fig.3B)

Combined PRP and Losartan Treatment Improved Muscle Strength

At 2 weeks post-injury, the PRP treated groups (PRP and PRP/losartan groups) showed significantly greater specific peak twitch and tetanic forces (twitch; 42.5±12.5 and 46.5±10.2, tetanic; 77.9±11.5 and 87.0±26.5g/cm^2, respectively) than the injured untreated contol group (twitch; 24.1±8.9 , tetanic; 47.6±7.26 and g/cm^2, respectively). (Fig. 4A)

The PRP/losartan group showed significantly greater specific peak twitch and tetanic forces (twitch; 60.2±11.2, tetanic; 112.2±25.9g/cm^2) than the other groups at 4 weeks after injury (control group: twitch; 24.4±5.1, tetanic; 59.1±13.3, losartan group: twitch; 39.2±3.0, tetanic; 81.9±23.2, and PRP group: 39.9±13.8, tetanic; 84.2±20.5 g/cm^2). Moreover, there was no significant difference between the PRP/losartan group and the uninjured normal group (twitch; 59.5±18.7, tetanic; 125.4±30.4g/cm^2). (Fig. 4B)

PRP Injection Enhanced VEGF Expression in Injured Muscle

VEGF expression areas in the injured TA muscles were measured and compared among the groups at 1 week post-injury (Fig 5A). The PRP treated groups (PRP, PRP/losartan groups) showed significantly greater VEGF immunoreactive areas (4.01±2.45% and 4.51±3.11%, respectively) when compared with the control and losartan groups (0.12±0.22 and 0.53±0.70 %, respectively). (Fig 5B).

PRP Injection Enhanced Angiogenesis in Injured Muscle

CD31expressing areas in the injured TA muscles were measured and compared among the groups at 1week post-injury (Fig 5C). The PRP injected groups (PRP, PRP/losartan groups) showed significantly greater areas of CD31 positive immunoreactivity (4.17±1.% and

5.18±1.61%, respectively) when compared with the control and losartan groups (0.94±0.82 and 1.38±1.29 %, respectively). (Fig 5D)

Administration of Losartan Suppressed pSmad2/3 Expression in Injured Muscle

The pSmad2/3 positive areas in the injured TA muscles were measured and compared among the groups at 2 weeks post-injury (Fig 6A). The losartan treated groups (losartan group; 0.82±1.15%, PRP/losartan group; 0.92±0.54%) showed significantly less pSmad2/3 positive areas than the control and PRP groups (4.22±1.57 % and 3.22±0.59 %, respectively). Moreover, the PRP group showed less pSmad2/3 positive areas than the injured untreated control group. (Fig 6B)

Combinatorial PRP and Losartan Treatment Enhanced FLST Expression in Injured Muscle

FLST expressing areas in the injured TA muscles were measured and compared among the groups at 2 weeks post-injury. The PRP/losartan group showed significantly greater FLST expression (5.99±2.74%) when compared with the other groups (control group; 0.47±0.32%, losartan group; 2.55±1.75%, PRP group; 3.87±1.72%) (Fig 7).

DISCUSSION

The current study evaluated the combinatorial use of both PRP injection and oral administration of losartan for treating muscle contusion injuries in mice. The aim of this study was to investigate and compare the beneficial effects of using PRP and losartan treatments alone and in combination for the treatment of contusion injured skeletal muscle . We hypothesized that 1) combining these treatments would lead to better improvements in muscle regeneration and muscle strength than administering PRP alone, and 2) blocking TGF-β1 by administering losartan in addition to PRP would reduce fibrosis formation in injured muscle and thereby further enhance the functional healing of the injured muscle.

During the healing process of injured skeletal muscle, the release of growth factors regulate muscle cell proliferation and differentiation to promote muscle regeneration and repair [25]. For skeletal muscle injury, several studies have reported that the injection of PRP can deliver many physiological growth factors and cytokines, at appropriate concentrations, to aid in the healing of injured skeletal muscle by activating satellite cells and ultimately enhancing muscle regeneration *in vivo* [23, 49]. Recent studies have shown that VEGF plays an important role in tissue healing through angiogenesis induction [35]. In injured skeletal muscle, the restoration of VEGF expression contributes to the dynamic process of capillary formation and muscle regeneration [39]. It has also been reported that FLST enhances myogenic differentiation and accelerates the maturation of myotubes in a dose dependent manner *in vitro*.[53] Our group (unpublished data, 2011) has recently demonstrated that losartan treatment can enhance the expression of FLST *in vivo*. We have also shown (unpublished data, 2010) that there is an abundance of FLST present in PRP and beleived that the use of PRP and losartan in combination could enhance the expression of both VEGF and FLST within injured muscle. Therefore, we posited that combining PRP and losartan treatments would improve muscle regeneration via the enhancement of VEGF and FLST expression.

TGF-β1 is a known potent fibrogenic cytokine and is considered a key factor in the development of pathological fibrosis [43, 50]. In our previous work, the oral administration of losartan was shown to inhibit fibrosis formation after skeletal muscle injury by blocking TGF-β1 [5]; however, the effect that PRP has on fibrosis formation in injured skeletal muscle is still unclear [22]. We believed that the TGF-β1 present in the PRP could have the potential to promote the formation of fibrosis. Our present results, however, demonstrated that the PRP groups had a reduction in the formation of fibrosis compared to the control group, though it did not completely inhibit fibrosis formation or the expression of pSmad2/3. Deasy et al reported that the implantation of VEGF overexpressing stem cells into injured skeletal muscle led to an increase in angiogenesis and a reduction in fibrosis [14]. In the current study, the PRP treatment groups showed

significantly greater VEGF expression than the untreated control group. Moreover, some studies have reported that FLST can stimulate angiogenesis *in vitro* and *in vivo* and decrease fibrosis formation, [29 37 52] which suggested that the injection of PRP into injured muscle could decrease fibrosis formation via the enhancement of angiogenesis through the increased expression of FLST, even though PRP contains high levels of TGF-β1.

The functional recovery of injured muscle is the most important outcome measure for patients who have suffered from a severe muscle injury [9]. Moreover, it has been reported that the improvements in muscle regeneration and the reduction of fibrosis correlate with enhanced muscle function [47]. Hammond et al showed that the injection of PRP in injured rat muscle could reduce the amount of time required to acheive full recovery after injury due to PRP's positive effect on myogenesis [23]. In the current study, physiological testing of the PRP treatment groups (PRP and PRP/losartan groups) showed more rapid improvements in muscle healing 2 weeks post-injury than the control and the losartan groups. Our results indicated that early recovery after PRP treatment might be promoted by an increase in angiogenesis at the injury site 1 week post-injury since we observed an enhancement of muscle regeneration at the 2 week timepoint. In addition, at 4 weeks post-injury, the PRP/losartan group demonstrated significantly greater muscle force than the PRP or losartan only groups. The physiological results corresponded to the histological results, which showed a complete inhibition of fibrosis in the PRP/losartan group suggesting that combining PRP and losartan treatments not only led to the rapid healing of the muscle histologically after 2 weeks, but more importantly, to the complete functional recovery of the muscle by 4 weeks post-injury. Indeed there was no statistical difference between the uninjured normal group and the PRP/losartan groups in their functional physiological capacity 4 weeks after injury.

Several limitations should be noted about the current study. The first limitation is that we have not examined the optimal timing, quantity or frequency of administering PRP. Most patients that suffer acute muscle injuries usually go to the hospital immediately or within 1 day after

experiencing an injury, thus from a clinical standpoint, PRP could be injected into the injured muscle by the 1st day after injury. With this in mind, the present study was designed so that PRP would be injected 1 day post-injury. PRP is currently used clinically for the treatment of muscle strain injury due to its safety and ease of administration [23]; therefore, we believe it would be quite simple to optimize the timing and dosing of a combined PRP/losartan therapy scheme for the treatment of skeletal muscle injuries in the near future. Although it was beyond the scope of the current study, the second limitation of this study was that it did not attempt to optimize the preparative technique for obtaining the best growth factor formulation of the PRP. It has been previously reported that the concentrations of growth factors in human PRP are very high [51]. The evaluation of human PRP isolated from the blood samples of 10 patients revealed that the concentrations of TGF-β1 were as high as what was observed in the current study (data not shown); however, we observed higher variability in the growth factor concentrations between the human patient samples than was observed in the current study. These findings are in agreement with data reported in the literature which demonstated growth factor concentration differences among PRP isolation protocols[11]. The literature also demonstrated the wide variability that exists among the individuals from which PRP is isolated, such as the variations in platelet number [11]; hence, it will be important to standardize the preparation procedures of PRP in the future.

In summary, we have demonstrated that a combinatorial PRP/losartan treatment regime could improve overall skeletal muscle healing after muscle contusion by increasing rapid revascularization of the injured muscle, enhancing muscle regeneration, and inhibiting the formation of fibrosis. More importantly,this combined treatment could accelerate the functional recovery of the muscle after injury. The greatest advantage of using this combinatorial treatment is that, 1) PRP is already used clinically for treating a variety of tissues including skeletal muscle, and 2) losartan is already FDA approved and has a relatively minor side effect profile. We believe

363 that these findings could contribute to the rapid development of clinically relevant biological
364 therapies for the treatment of skeletal muscle injuries.

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Figure 1.

Schematic diagram showing the experimental design.

Figure 2.

A. Histological evaluation of muscle regeneration at 2 weeks post-injury by H&E staining of the TA muscles treated with PRP and/or losartan (control: normal drinking water and injection of PBS; losartan: losartan supplemented drinking water and injection of PBS; PRP: normal drinking water and injection of PRP, losartan/PRP: losartan supplemented drinking water and injection of PRP). Regenerating myofibers were identified by locating centronucleated myofibers (original magnification, ×200).

B. The graph depicts quantification of the number of regenerating myofibers in PRP and/or losartan treated animals compared with the other animals. hpf, high-power field. *$p<0.05$ to the control group. **$p<0.05$ to the control and losartan groups. *** $p<0.05$ to the other groups.

Figure 3.

A. Histological evaluation of fibrosis formation at 4weeks post-injury by Masson's trichrome staining (collagen, blue; myofibers, red; nuclei, black) of the TA muscles treated with PRP and/or losartan. The ratio of the fibrotic area to the total cross-sectional area of 10 randomly selected sections was calculated to estimate fibrosis formation (original magnification, ×200).

B. The graph depicts quantification of the fibrotic area in animals treated with PRP or losartan or with both PRP and losartan compared with the other animals.*$p<0.05$ to the control groups. **$p<0.05$ to the control and PRP groups.

Figure 4.

A-B. Functional recovery was evaluated in each group by physiological testing at 2 and 4 weeks after muscle injury. The graph depicts the specific peak twitch force (A) and the specific peak tetanic force (B) in animals treated with PRP and/or losartan. (control: white bars; losartan: light gray bars; PRP:dark gray bars; losartan/PRP: black bars) *$p<0.05$ to the control groups at 2

weeks, **p<0.05 to the control group at week 4, and *** p<0.05 to the other groups at 4 weeks, except the normal group.

Figure 5.

A. Immunohistochemical staining of tissue samples collected at 1 week post-injury with anti-mouse VEGF antibody (red fluorescence) demonstrated enhanced expression of VEGF around the injury site in muscles treated with PRP or without losartan treatment (original magnification, ×200).

B. The graph depicts quantification of VEGF expression in animal treated with PRP or losartan or with both PRP and losartan compared with the control animals. *p < 0.05 to the control group. **p<0.05 to the control and losartan groups.

C. Immunohistochemistry of the tissue samples demonstrated that angiogenesis was detected at the peri-injury site in the PRP treated groups at 1 week post-injury. Mouse endothelial cells were detected with an anti-CD31 Ab (red fluorescence) and the nuclei via DAPI staining (blue fluorescence), respectively (original magnification, ×200).

D. The graph depicts quantification of the areas of CD31 positivity in animals treated with PRP or losartan or with both PRP and losartan compared to the control animals. *p < 0.05 to the control and losartan groups.

Figure 6.

A. Immunohistochemical staining of tissue samples collected at 2 weeks post-injury with anti-mouse pSmad2/3 antibody (green fluorescence) demonstrated enhanced expression of pSmad2/3 around the injury site in muscles treated with PRP and/or losartan (original magnification, ×200).

B. The graph depicts quantification of pSmad2/3 expression in animals treated with PRP or losartan or with both PRP and losartan compared to the control animals. *p < 0.05 to the control group. **p<0.05 to the control and PRP groups.

Figure 7.

601 The graph depicts quantification of FLST expression in animals treated with PRP losartan or with
602 both PRP and losartan compared to the control animals. *$p < 0.05$ to the control group. **$p<0.05$
603 to the other groups.

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Figure 1.

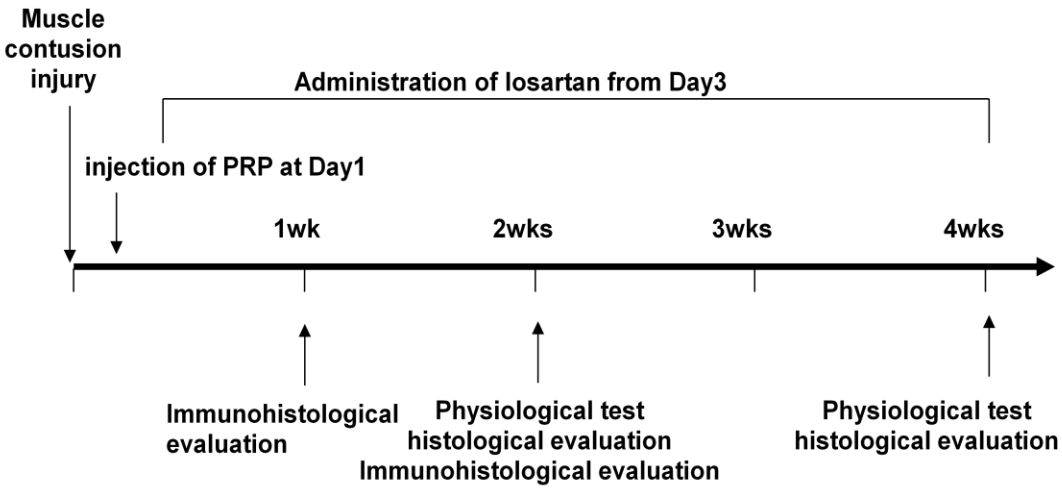
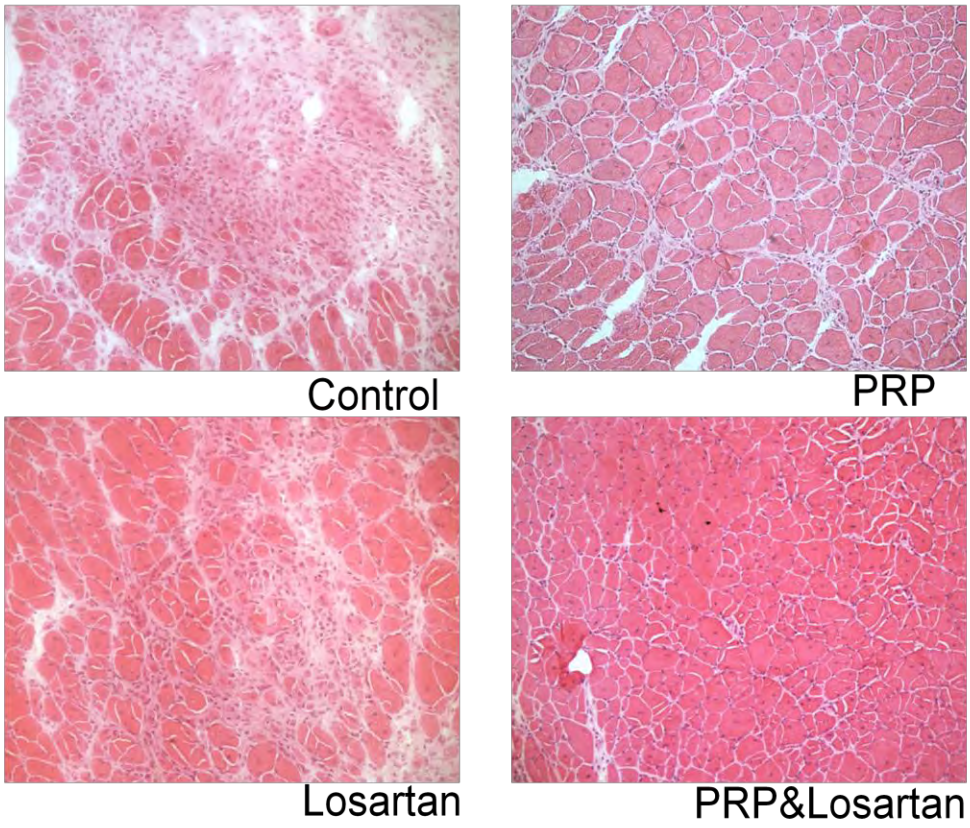

Muscle contusion injury
Administration of losartan from Day3
injection of PRP at Day1
1wk
2wks
3wks
4wks
Immunohistological evaluation
Physiological test
histological evaluation
Immunohistological evaluation
Physiological test
histological evaluation

Figure 2.

A



B

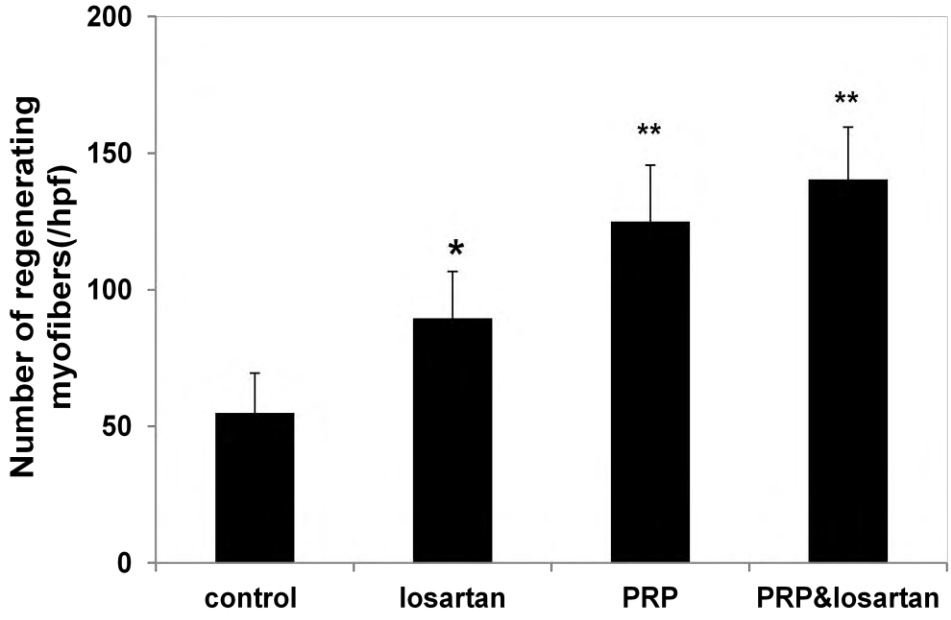


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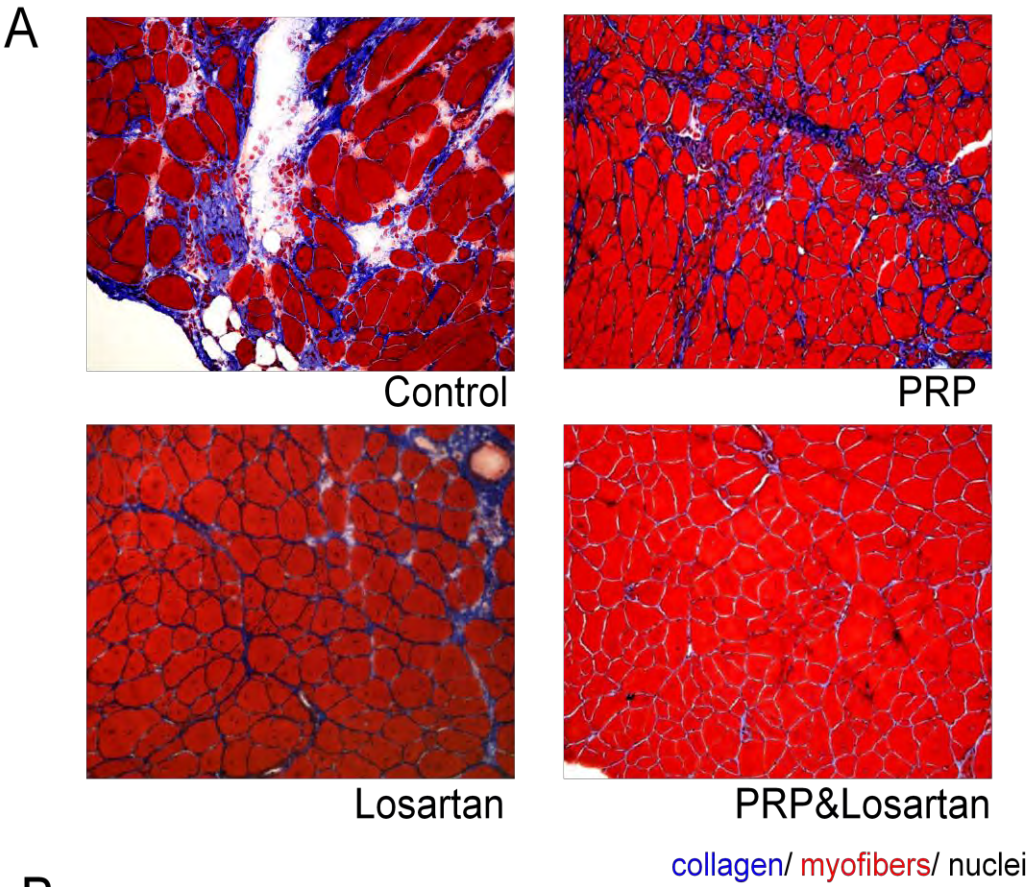
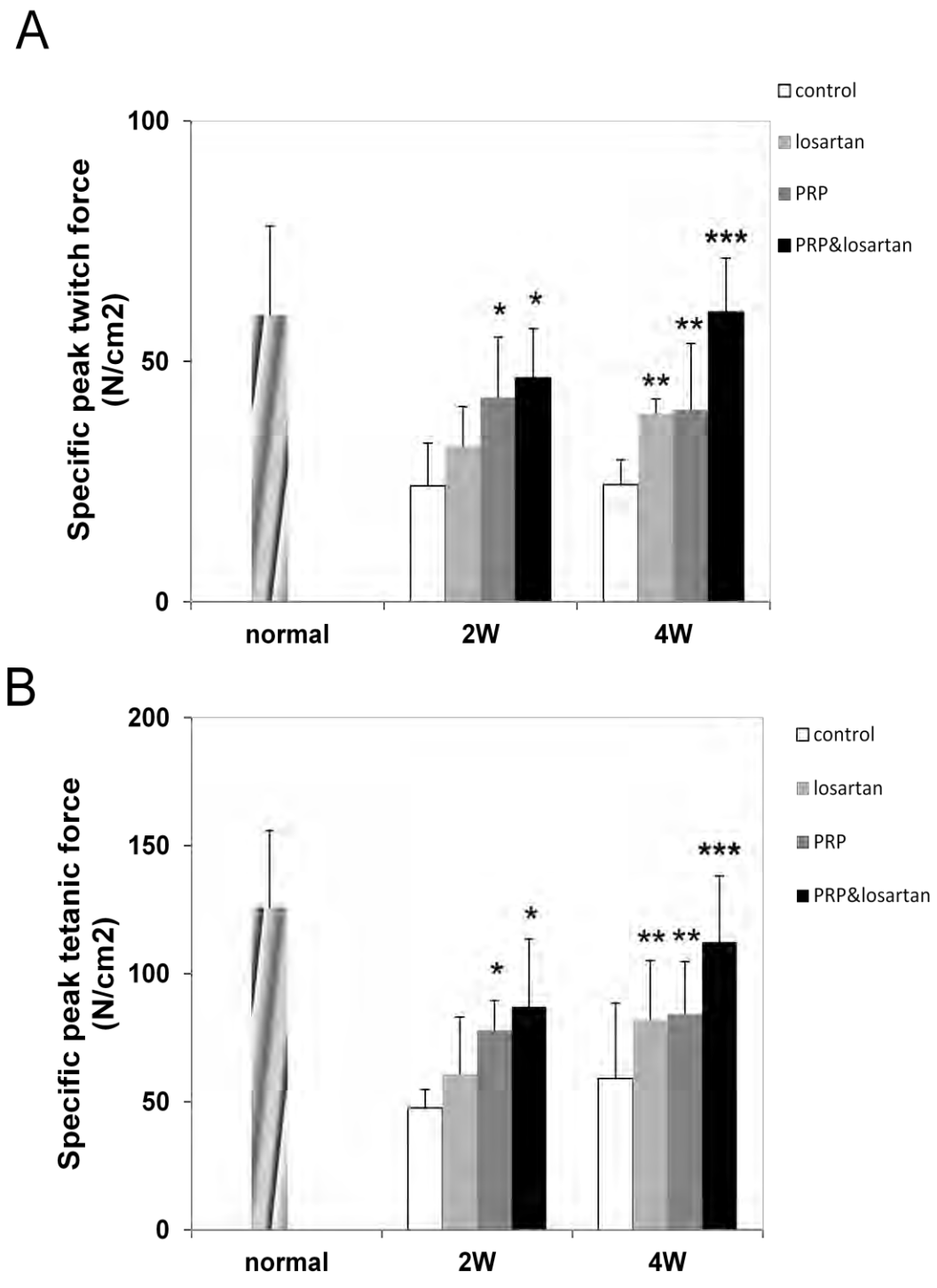
Figure 3.


A
Control
PRP
Losartan
PRP&Losartan
collagen/ myofibers/ nuclei
B
Fibrotic area (%)
30
25
20
15
10
5
0
**
*
**
control
losartan
PRP
PRP&losartan

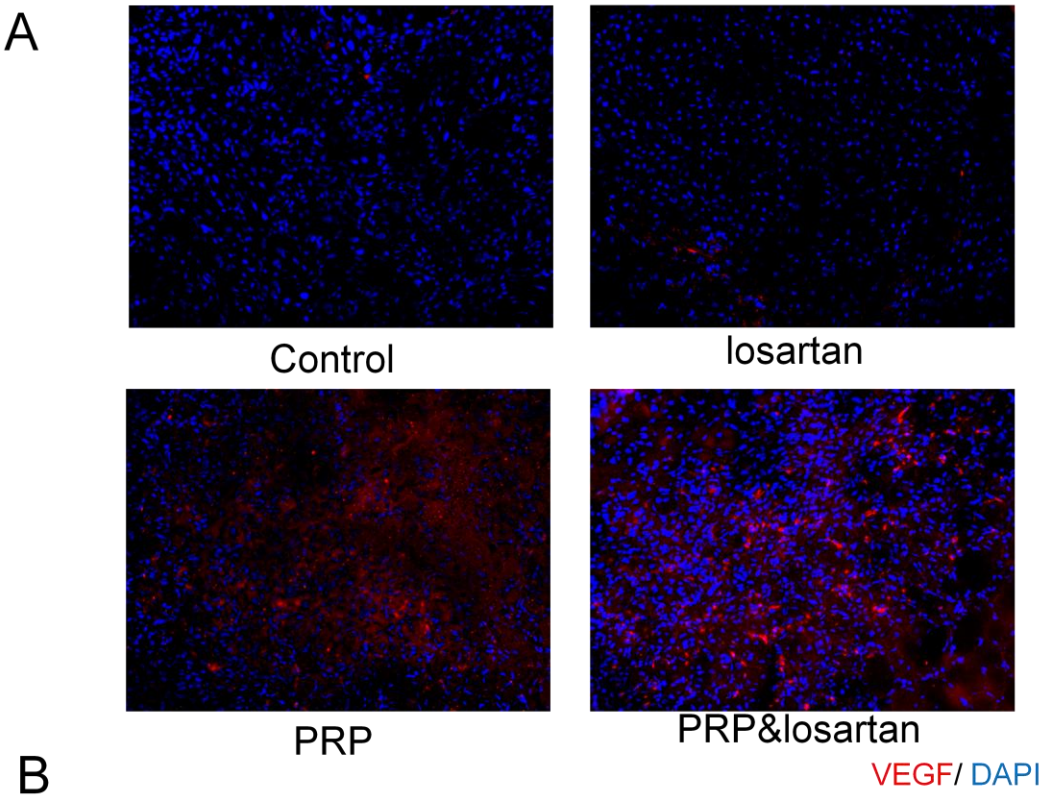
624

Figure 4.


A
control
losartan
PRP
PRP&losartan
Specific peak twitch force (N/cm2)
100
50
0
*
*
**
**
***
normal
2W
4W
B
control
losartan
PRP
PRP&losartan
Specific peak tetanic force (N/cm2)
200
150
100
50
0
*
*
**
**
***
normal
2W
4W

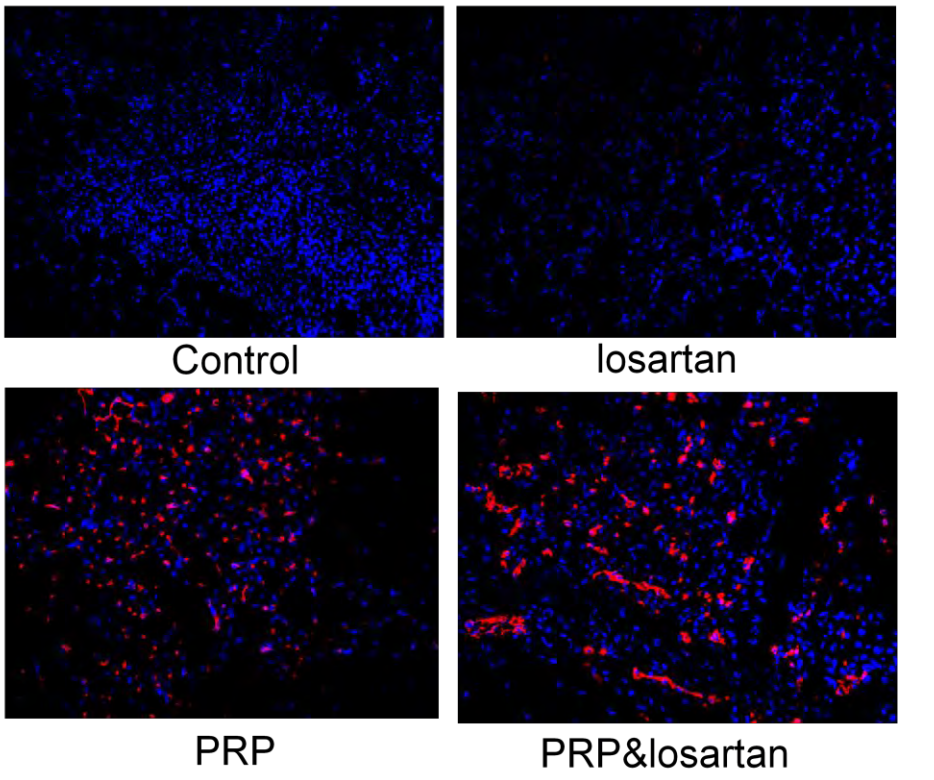
625

Figure 5.

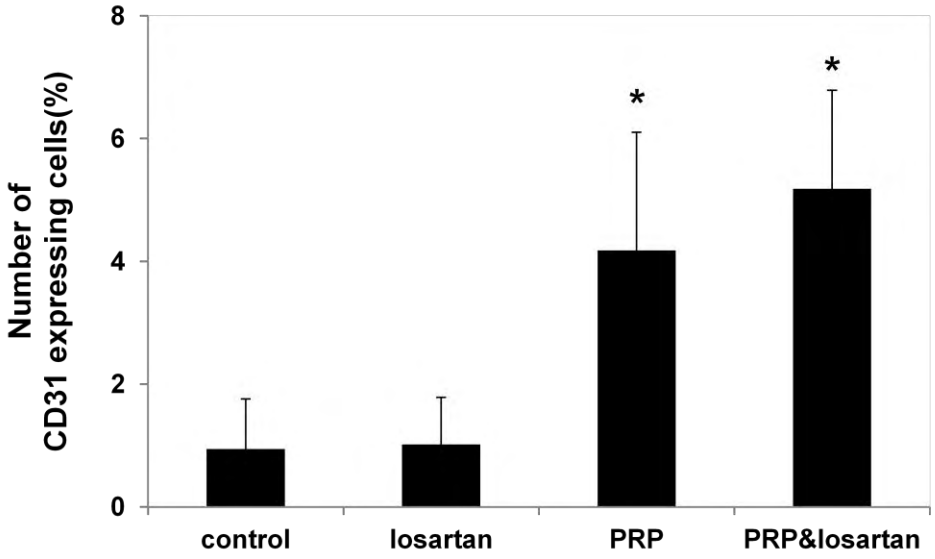

A
Control
losartan
PRP
PRP&losartan
VEGF/ DAPI
B
Expression of VEGF (%)
8
6
4
2
0
*
*
control
losartan
PRP
PRP&losartan

626

C



D



627
628
629
630
631

Figure 6.

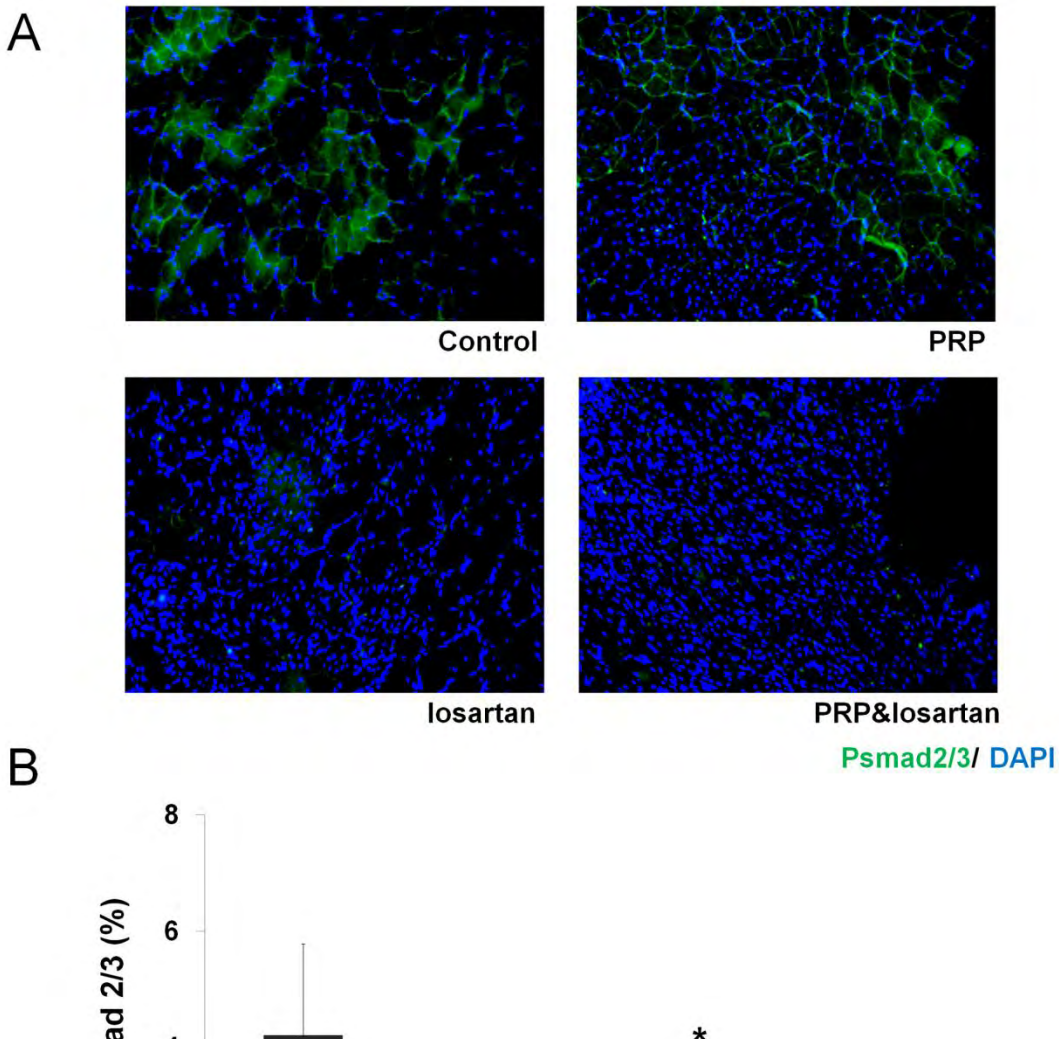

A
Control
PRP
losartan
PRP&losartan
Psmad2/3/ DAPI
B
Area of pSmad 2/3 (%)
8
6
4
2
0
**
*
**
control
losartan
PRP
PRP&losartan

Figure 7.

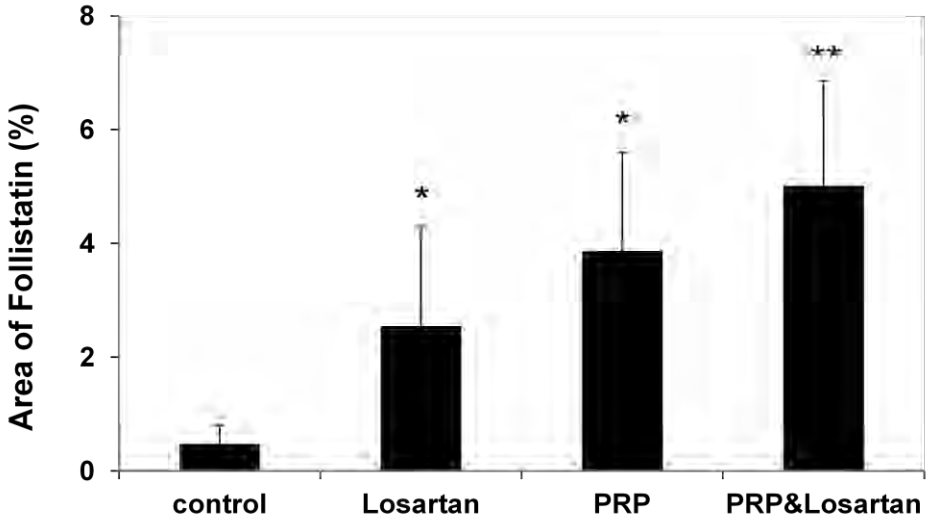


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Table 1: immunohistochemistry staining protocols				
Stain	**Vascular endothelial growth factor (VEGF)**	**CD31**	**pSmad2/3**	**Follistatin (FLST)**
Fixation	Methanol (5 min)	Methanol (5 min)	Methanol (5 min)	Methanol (5 min)
Blocking Reagents	10% Horse Serum (HS)(60 min; Invitrogen)	10% Horse Serum (HS)(60 min; Invitrogen, Carlsbad, CA)	10% Horse Serum (HS)(60 min; Invitrogen)	10% Horse Serum (HS)(60 min; Invitrogen)
1°	Rabbit anti-mouse VEGF (1:100 in 2.5% DS; Overnight at 4 ºC; Abcam)	Rat anti-mouse CD31 (1:100 in 2% HS; overnight at 4°C; BD Pharmingen, Franklin Lakes, NJ)	Goat anti-mouse p-Smad2/3 (1:300 in 2% HS; overnight at 4°C; Santa Cruz, Santa Cruz, CA)	Goat anti-mouse follistatin (1:100 in 2% HS; overnight at 4°C; Santa Cruz, Santa Cruz, CA)
2°	Anti-rabbit IgG-Alexa Fluor 594 (1:300 in 2% HS; 60 min; Molecular Probes)	Anti-rat IgG-Alexa Fluor 594 (1:300 in 2% HS; 60 min; Molecular Probes)	Anti-goat IgG-Alexa Fluor 488 (1:300 in 2% HS; 60 min; Molecular Probes)	Anti-goat IgG-Alexa Fluor 488 (1:300 in 2% HS; 60 min; Molecular Probes)
3°	4, 6-Diamidino-2-phenylindole (DAPI) (1:1000 in PBS; 5 min, Sigma)	4, 6-Diamidino-2-phenylindole (DAPI) (1:1000 in PBS; 5 min, Sigma)	4, 6-Diamidino-2-phenylindole (DAPI) (1:1000 in PBS; 5 min, Sigma)	4, 6-Diamidino-2-phenylindole (DAPI) (1:1000 in PBS; 5 min, Sigma)

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Biological approaches to improve skeletal muscle healing after injury and disease

Running title: *Modulating skeletal muscle repair by muscle derived stem cells and antifibrotic agents*

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Abstract

Skeletal muscle injury and repair are complex processes including well-coordinated steps of degeneration, inflammation, regeneration and fibrosis. We reviewed recent literature including studies by our group that describe how to modulate the processes of skeletal muscle repair and regeneration.

Anti-inflammatory drugs such as cyclooxygenase-2 were found to hamper the skeletal muscle repair process. Muscle regeneration phase can be aided by growth factors including IGF-1 and NGF but these factors are typically short-lived and thus more effective methods of delivery are needed.

Skeletal muscle damage caused by traumatic injury or genetic diseases can benefit from cell therapy. However, the majority of transplanted cells are unable to survive the immune response and hypoxic conditions. Our group has isolated neonatal skeletal muscle derived stem cells (MDSCs) which seem to repair muscle tissue in a more effective manner than myoblasts probably due to better resistance to oxidative stress. Enhancing antioxidant levels of MDSCs lead to improved regenerative potential.

It is becoming increasingly clear that stem cells direct tissue repair by direct differentiation and paracrine effects leading to neovascularization of injured site and chemoattraction of host cells. The factors invoked in paracrine action are still under investigation.

Our group has found that angiotensin II receptor blocker (losartan) significantly reduces fibrotic tissue and improves repair of murine injured muscle. Based on these data, we have conducted a case study on two hamstring injury patients and found that losartan treatment promoted faster muscle recovery. We believe this medication holds great promise to optimize muscle repair in humans.

Introduction:

Vertebrate skeletal muscle is composed of bundles of contractile muscle fibers which are multinucleated cells formed by the fusion of muscle cells (myocytes) into syncytia. The myofibers are surrounded by a membrane (sarcolemma) and each group forms a fascicle. Fascicles are in turn surrounded by connective tissue sheath called the endomysium. Groups of fascicles are enveloped by a perimysium and make up whole muscle. The skeletal muscle also contains connective, vascular, adipose, nerve and other cell populations.

The myogenic cells in the skeletal muscle are found at a variety of different stages of maturity. The fully differentiated myofibers are surrounded by satellite cells that are within the basal lamina but outside the sarcolemma (Mauro, 1961). These cells express paired box protein 7 (Pax7) and MyoD surface markers, which indicate partial differentiation down the muscle lineage (Seale et al., 2000). Satellite cells remain quiescent in the muscle until an external stimulus such as injury occurs, at which point they re-enter the cell cycle and proliferate (Hill et al., 2003). Proliferating satellite cells differentiate into myoblasts, which can then fuse to form new myofibers (Collins and Partridge, 2005). The myoblast population, which is more differentiated than satellite cells, fuse to become mature myofibers, as previously mentioned. These cells have also been used extensively for transplantation.

Distinct from both the satellite cell and myoblast populations are the muscle derived stem cells (MDSCs). Although MDSCs are similar to satellite cells in their regeneration abilities in skeletal muscle, they are a separate population of cells that express distinct markers and phenotypes (Deasy et al., 2001; Huard et al., 2003). MDSCs are believed to an earlier progenitor than satellite cells, expressing stem cell markers such as cluster of differentiation 34 (CD34) and stem cell antigen 1 (Sca-1), and have the ability to differentiate down non-muscle lineages to contribute to repair (Qu-Petersen et al., 2002). Pax-7 and Sca-1 positive cells have not been co-localized in skeletal muscle, providing further evidence that satellite cells and MDSCs are likely distinct populations (Zammit and Beauchamp, 2001). **Figure 1.**

In the incidence of a traumatic injury or muscle damage due to a genetic disease (such as Duchenne Muscular Dystrophy, DMD), the muscle goes through a complex and dynamic series of events resulting in an inflammatory phase, the activation of progenitor cells, regeneration of muscle tissue, formation of fibrosis and varying degrees of restoration of function. These phases are detailed below.

Phases of skeletal muscle injury and repair

Acute skeletal muscle injuries are common injuries which account for a large segment of the patients presenting to orthopaedic practitioners (Carling et al., 2011; Crisco et al., 1994; Physicians; Woolf and Pfleger, 2003). Research has shown that the natural progression of muscle injury proceeds through a highly interdependent sequence of steps, leading to the restoration of normal tissue architecture and function (Moyer and Wagner, 2011). However, the regenerative capacity of injured skeletal muscle is limited and very often, fibrotic tissue forms, delaying the muscle's full functional recovery and predisposing it to recurrent injury (Li and Huard, 2002). Clinical data reveals a high recurrence rate of skeletal muscle strain injuries, approaching 30% among some professional-level athletes (Woods et al., 2004). Numerous investigations led to the identification of molecular events and cellular transformations following muscle injury; however, the clinical treatment of this common condition still relies upon conventional therapies of rest, ice, and anti-inflammatory medications, which have a limited efficacy in preventing or treating the formation of post-traumatic muscle fibrosis (Almekinders, 1993; Jarvinen and Lehto, 1993; Worrell, 1994). Research conducted by our group and others showed that injured muscle undergoes a sequential process of healing phases, including muscle degeneration/inflammation, regeneration, and fibrosis (Huard et al., 2002; Li et al., 2004; Li and Huard, 2002). These phases of muscle healing can be modulated by different biological approaches that will be detailed in the sections below. **Figure 2.**

Muscle Inflammation:

Muscle degeneration and concomitant inflammation begins in the first few days post injury. Resident macrophages are activated releasing chemoattractants leading to the

recruitment of neutrophils and monocytes. Subsequently, inflammatory mediators such as tumor necrosis factor a (TNFα) are released and immune, myogenic, and fibroblastic cell interactions are coordinated. This reaction can persists for several days depending on the severity of the injury (Moyer and Wagner, 2011).

Anti-inflammatory drugs are often prescribed to relieve pain after muscle injury. However, the effect of these drugs, especially non-steroidal anti-inflammatory (NSAIDs) on the skeletal muscle healing remains controversial. To examine the role NSAIDs play in the process of muscle healing, our group had performed two studies to determine the effect that cyclooxygenase-2 (Cox-2) in modulating muscle recovery (Shen et al., 2005; Shen et al., 2006). In vitro experiments showed that a Cox-2-specific inhibitor (NS-398) slows the proliferation and maturation of differentiated myogenic precursor cells and thus delays the regenerative myogenesis process. Other investigators have found similar results using the Cox-2 selective inhibitor SC-236 (Bondesen et al., 2004; Bondesen et al., 2006). Our results thus indicate that NS-398 may hamper skeletal muscle healing. We examined the in vivo effect of NS-398 on skeletal muscle healing in a muscle laceration mouse model at different time points up to 4 weeks post injury. The in vivo data were in agreement with in vitro results and showed delayed muscle regeneration at early time points after injury in the NS-398 treated mice. Treating lacerated muscles with NS-398 lead to the expression of higher levels of transforming growth factor-β1 (TGF-β1) than the untreated control muscles and the lacerated muscles treated with NS-398 showed higher fibrosis deposition than the controls. As expected we found fewer neutrophils and less macrophage infiltration in the muscles treated with NS-398. These results indicate that the inhibitory effect of NS-398 on the inflammatory responses delays skeletal muscle healing after laceration. Furthermore, we analyzed muscle healing following laceration injury on the tibialis anterior (TA) muscles of COX-2-/- mice and control wildtype (Shen et al., 2005) by examining the histology and function of TA muscles at 5 and 14 days after injury. COX-2-/- mice TA muscles showed decreased regeneration relative to that observed in wildtype mice. These results demonstrate that the COX-2 pathway plays an important role in muscle healing and consequently the decision to use NSAIDs to treat muscle injuries warrants critical examination of evidence available. NSAIDs seem to impair the healing even in

the heavily vascularized skeletal muscle tissue and probably affect the recovery of other soft tissues.

Muscle Regeneration:

In the first week post injury, skeletal muscle promptly begins the regeneration process which peaks at 2 weeks, and then decrease gradually 3-4 weeks post injury. Several studies have shown that growth factors play a variety of roles during muscle regeneration (Allen and Boxhorn, 1989; Anderson et al., 1991; Barnard et al., 1994; Barton-Davis et al., 1998; Chambers and McDermott, 1996; Coleman et al., 1995; Damon et al., 1998; De Deyne et al., 2002; Doumit et al., 1993; Engert et al., 1996; Florini et al., 1996; Florini et al., 1986; Floss et al., 1997; Gospodarowicz et al., 1976; Gowdak et al., 2000; Grounds, 1991; Harrington et al., 1992; Inselburg and Applebaum, 1978; Jennische, 1989; Jin et al., 1990; Johnson and Allen, 1995; Jones and Clemmons, 1995; Keller et al., 1999; Kurek et al., 1997; Lamberts et al., 1997; Lefaucheur and Sebille, 1995; Linkhart et al., 1981; McFarland et al., 1993; Musaro et al., 2004; Olson et al., 1986; Papadakis et al., 1996; Quinn and Haugk, 1996; Sheehan et al., 2000; Springer et al., 1998; Tatsumi et al., 1998; Wieteska-Skrzeczynska et al., 2011a; Wieteska-Skrzeczynska et al., 2011b; Yablonka-Reuveni et al., 1990; Zdanowicz et al., 1995). Using a mouse model, Menetrey et al. found that direct injections of insulin-like growth factor-1 (IGF-1), basic fibroblastic growth factor (bFGF), and, to a lesser extent, nerve growth factor (NGF), lead to enhanced muscle healing in lacerated, contused, and strain-injured muscle at 2, 5, and 7 days after injury (Menetrey et al., 2000). Using human recombinant growth factors to treat muscle injuries has the advantage of feasibility and safety of the injection; however, the efficacy of direct injection of growth factors made from recombinant proteins is limited by the high concentrations typically required to elicit a measurable effect. Growth factors clearly have a dose-dependent effect on myoblast proliferation and differentiation in vitro. However, a series of 3 consecutive injections of a relatively high concentration (100 ng/growth factor) of each of NGF, IGF-1, or bFGF factors are usually necessary to achieve significant enhancement of skeletal muscle healing in mice (Barton-Davis et al., 1998; Chan et al., 2003; Foster et al., 2003; Fukushima et al., 2001; Li et al., 2004; Li and Huard, 2002; Menetrey et al., 2000; Negishi et al., 2005; Sato et al., 2003). Rapid

clearance of these molecules by the blood stream and their relatively short biological half-lives necessitate such large concentrations to be typically administered. Further studies will be needed to investigate the potential synergetic effects of the association of two or more growth factors or the potential use of controlled release particles containing the factors.

Another method that can be effective in delivering high, maintainable concentrations of growth factors to injured muscle is gene therapy. Data obtained from previous studies had demonstrated that IGF-1 is a potent growth factor for stimulating muscle regeneration and improving muscle healing in vivo after injury (Barton-Davis et al., 1998; Chan et al., 2003; Foster et al., 2003; Fukushima et al., 2001; Li et al., 2004; Li and Huard, 2002; Menetrey et al., 2000; Negishi et al., 2005; Sato et al., 2003). Based on this information, we genetically engineered an adenoviral vector to encode the gene for IGF-1 and evaluated its ability to improve muscle healing after injury (Lee et al., 2000). Myoblasts ex vivo transduced by IGF-1 adenovirus and then injected into lacerated muscles of immunocompetent mice lead to improved muscle repair (Lee et al., 2000). Although we have seen improvement in muscle strength in skeletal muscle treated with myoblasts adenovirally treated to express IGF-1, fibrosis or scar tissue was detected by histology. This suggested that the enhancement of muscle regeneration by either cell or gene therapy improves muscle regeneration but may not completely eliminate fibrosis (Lee et al., 2000). These results suggest that high levels of IGF-1 secretion can be achieved and delivery can is feasible but the functional recovery of the injured muscle remained incomplete. It is likely that IGF-1 modulates several actions including a stimulatory effect on myofibroblast proliferation, deposition of extra cellular matrix (ECM) which may interfere with the ability of this growth factor, even at high concentrations, to improve muscle healing after injury (De Deyne et al., 2002; Jones and Clemmons, 1995). Overall, these results indicate that reducing muscle fibrosis is a complicated process that likely involves several cell populations and growth factors.

Muscle regeneration after stem cell transplantation

A variety of muscle cell populations have been used for cell transplantation in studies treating patients who are afflicted with DMD, a muscle disease characterized by the lack

of dystrophin expression at the sarcolemma of muscle fibers (Hoffman et al., 1987). Transplantation of committed myoblasts into dystrophin-deficient muscle delivers normal myoblasts that fuse with host muscle fibers and/or among themselves and consequently restores dystrophin expression; however, this approach is hindered by numerous limitations including limited cell spreading, immune responses, and poor survival of the transplanted cells (Fan et al., 1996; Gussoni et al., 1997; Huard et al., 1991; Huard et al., 1994; Karpati et al., 1989; Mendell et al., 1995; Morgan et al., 1990). Although the immune response and the low spreading capacity of the cells have been overcome, at least in part (Kinoshita et al., 1994; Vilquin et al., 1995), the low survival of the transplanted cells is still a major limitation. Indeed, numerous studies report that only a small percentage of the transplanted cells (less than 1-5%) survive, therefore, approaches to increase cell survival post-implantation need to be optimized for myoblast transplantation therapies for DMD (Beauchamp et al., 1999; Fan et al., 1996; Hodgetts et al., 2000).

Efforts to promote donor myoblast survival initially focused on overcoming the inflammatory response (Guerette et al., 1997; Hodgetts et al., 2000; Qu et al., 1998). Myoblasts genetically engineered to express an inhibitor of the inflammatory cytokine, IL-1, showed an improved survival rate compared to non-engineered cells (Qu et al., 1998). By treating the host with CD4+/CD8+ to deplete antibodies, donor myoblast survival was enhanced in dystrophic animals (Hodgetts et al., 2000), and by treating the host animals with antibodies against Leukocyte function-associated molecule 1 (LFA-1), death of the transplanted myoblasts was reduced (Guerette et al., 1997). This has lead some investigators to focus their efforts on the isolation, identification, and characterization of the small subset of donor cells capable of surviving after transplantation (Baroffio et al., 1996; Beauchamp et al., 1999; Collins et al., 2007). Our efforts to isolate such a population are detailed in the following section.

Muscle derived stem cells

Our group and others have isolated from mouse skeletal muscle a group of cells based on their adhesion characteristics to collagen coated flasks. We used a modification of a method called the preplate technique (Gharaibeh et al., 2008; Qu-Petersen et al., 2002)

to purify these cells from mouse skeletal muscles. In the preplating process, a skeletal muscle biopsy is mechanically broken and then enzymatically digested by a series of enzymes including dispase, collagenase, and trypsin. A single cell suspension is filtered and plated onto a series of collagen-coated flasks. The cells that are slowest to adhere (Slowly Adhering Cells; SAC) seem to proliferate very slowly at first and have different morphology from the rapidly adhering fraction (RAC). After further passaging to remove other cells including fibroblasts and myoblasts, SAC becomes highly enriched with in muscle-derived stem cells (MDSCs). Technical details of the protocol followed to isolate MDSCs are beyond the scope of this review (Gharaibeh et al., 2008). MDSCs have the potential for long term proliferation without any significant changes to their cell characteristics, and they maintain a stable karyotype that has no significant numerical or structural abnormalities (Deasy et al., 2005). The end result of the preplate technique results in an enriched population of MDSCs, nevertheless, it is a heterogeneous population. A marker profile for MDSCs is not very high for a single marker as one would obtain from isolation done by flow activated cell sorting (FACS), but typically MDSCs express high levels of Sca-1, as previously mentioned, very low levels of vimentin (a fibroblastic marker) and have low expression of desmin and other differentiated muscle markers (Qu-Petersen et al., 2002).

We have shown that SAC repairs skeletal muscle in a more effective manner than myoblasts which tend to more rapidly adheres to collagen coated flasks. Other investigators have shown that a sub-population of muscle cells are slowly dividing (as determined by thymidine uptake) but when injected into dystrophic mice muscles, they undergo rapid proliferation and become major contributors to muscle repair (Beauchamp et al., 1999). Similarly, Collins et al showed that among aged muscle satellite cells, there is also a subset of cells that survive the effects of aging and this minority population is responsible for muscle regeneration while the majority of cells progress to apoptosis; (Collins et al., 2007). Recent studies used FACS to separate cells based on normally expressed cell-surface markers and showed that skeletal muscle precursor cells (SMPs) show heterogeneity (Biressi and Rando, 2010; Cerletti et al., 2008). The subfraction characterized by markers $CD45^{-}Sca\text{-}1^{-}Mac\text{-}1^{-}CXCR4^{+}\beta1\text{-}integrin^{+}$ showed a high level of muscle cell repair, while non-SMPs ($CXCR4^{-}$ /β1-

integrin^{-}) were rarely identified in the muscles after transplantation (Cerletti et al., 2008). One can speculate that these subpopulations secrete different factors and have autocrine and paracrine effects on the host skeletal muscle and other tissues. These signals probably support the survival of these progenitor cells and enhance their participation in skeletal muscle repair. Indeed, we have found in previous studies that the transplantation of MDSCs that were transduced with a retroviral vector to express nerve growth factor (NGF) or directly stimulated with NGF protein, into the skeletal muscle of dystrophic mdx mice, resulted in a significantly larger engraftment with a higher number of dystrophin-positive myofibers than the transplantation of non-transduced MDSCs (Lavasani et al., 2006). Our observations of newly regenerated myofibers by the transplanted MDSCs, particularly the genetically engineered MDSCs, suggest that NGF released had an autocrine as well as paracrine effect on neighboring cells.

One important factor that may be involved in cell survival and tissue regeneration is the cells' expression of antioxidants. It was demonstrated in recent studies that a reduction of antioxidant levels negatively affects the regeneration index of myoblasts and satellite cells (Fulle et al., 2005; Lee et al., 2006; Urish et al., 2009), and hematopoietic stem cells (Ito et al., 2006), likely through the cells' increased ability to survive after implantation. Our group has recently shown that the MDSCs express high levels of the antioxidants glutathione (GSH) and superoxide dismutase (SOD). These molecules likely play a critical role in the cells' ability to survive the harsh transplantation microenvironment better than myoblasts and hence increase their ability to more efficiently regenerate the tissue (Drowley et al., 2010; Urish et al., 2009). Our group has recently shown that reduction of the antioxidant level of MDSCs by diethyl maleate decreased their regeneration potential while an improvement in their regenerative potential was observed after enhancing their antioxidant levels with N-acetylcysteine (NAC). These results show a therapeutic potential for boosting antioxidant levels of stem cells prior to transplantation in skeletal and heart muscles (Drowley et al., 2010; Urish et al., 2009).

Donor cell-mediated skeletal tissue repair paracrine action

It is clear from numerous reports using cell therapy in animal models that cell survival, differentiation and engraftment in host tissue are important factors in assessing the efficacy of cell therapy. At the same time, it has been demonstrated that the number of cells directly involved in tissue repair are not necessarily correlated with the improvement in function exhibited by the host tissue. This is especially true in the cardiac muscle (Payne et al., 2005). Recently, our group has found that intramuscular injection of MDSCs at 4 days post injury greatly enhanced injured skeletal muscle healing by increasing angiogenesis and reducing scar tissue formation (Ota et al., 2011). High levels of expression of vascular endothelial growth factor (VEGF) 1 week after cell injection was correlated with increased vascularity, improved muscle regeneration and strength at week 4 (Ota et al., 2011). Thus, it is believed that the ability of MDSCs to secrete different molecules and engage in autocrine and paracrine activities are determining factors of the success of these stem cells in the repair process (Gharaibeh et al., 2011). Exciting results now show that stem cell-mediated repair may occur with little if not the absence of donor cell differentiation. Results indicate that repair may be due to a largely overlooked characteristic of stem cells –paracrine signaling by surviving stem cells (Gharaibeh et al., 2011; Gnecchi et al., 2005; Murry et al., 2006). **Figure 3.**

Trophic signaling or release of cytokines or other signaling molecules may might be a key for recruiting host cells to participate in the repair, perhaps by having an effect in the local micro-environment and/or by inducing a systemic effect or by mediating an inflammatory response. Several report have shown that transplanted muscle cells may induce angiogenesis in the host tissue by expressing VEGF but it is not fully understood if other bioactive factors may be secreted by the cells nor whether they have any immunomodulatory activity, (Deasy et al., 2009; Springer et al., 1998; Springer et al., 2003). On a similar note, evidence indicating that anti-inflammatory drugs delay muscle repair implicates inflammatory cells in the repair process but what particular host cells that may play a role in the repair process is still unclear (Almekinders and Gilbert, 1986; Mishra et al., 1995; Obremsky et al., 1994; Shen et al., 2005).

Based on these findings, further analysis of the identity of specific molecules expressed in this paracrine activity will likely affect therapeutic strategies and indeed the need for stem cell transplantation.

Fibrosis:

Excessive formation of connective tissue between skeletal muscle fibers (or muscle scar) usually begins between the second and third week after muscle injury, and continues to increase in size over time (Huard et al., 2002; Li et al., 2001). Our findings strongly indicate that scar tissue formation leads to incomplete regeneration of injured muscle tissue. Various reports have implicated TGF-β1 in the onset of fibrosis in various tissues (Barcellos-Hoff et al., 1994; Barnes and Abboud, 1993; Brandes et al., 1991; Coimbra et al., 1991; Czaja et al., 1989; Kagami et al., 1994; Khalil et al., 1993; Logan et al., 1994; Okuda et al., 1990; Sporn and Roberts, 1993; Westergren-Thorsson et al., 1993; Wolf et al., 1994; Wynn, 2007; Yamamoto et al., 1993). However, very few studies have examined the direct role of TGF- β1 in skeletal muscle fibrosis (Fanbin et al., 2011). It was shown that TGF-β1 is expressed at high levels and is associated with fibrosis in the skeletal muscle of Duchenne muscular dystrophy (DMD) patients (Bernasconi et al., 1995; Cohn et al., 2007; Yamazaki et al., 1994; Zanotti et al., 2005). Research has shown significantly higher levels of TGF-β1 mRNA levels in muscle biopsy specimens of patients with dermatomyositis (Amemiya et al., 2000; Confalonieri et al., 1997). The studies concluded that excessive TGF-β1 is correlated with chronic inflammation, the accumulation of ECM, and fibrosis (Amemiya et al., 2000; Confalonieri et al., 1997). Members of TGF-β superfamily have been shown to be involved in Marfan syndrome and other inherited or acquired myopathies (Burks and Cohn, 2011; Cohn et al., 2007). We have used immunohistochemistry to study the expression of TGF-β1 and found strong expression of this cytokines in injured skeletal muscles in mice (Li et al., 2004). Taken together, these results clearly implicate TGFβ-1 in initiating a cascade of events that occur after muscle trauma or with the onset of muscle disease and suggest that neutralizing TGFβ-1 or down regulating its expression could eliminate or reduce scar formation.

Modulating fibrosis with anti-fibrotic agents

Our group has accumulated evidence from recent studies that showed that inhibiting the expression of TGF-β1 by using several anti-fibrotic agents lead to reduced muscle fibrosis. Using decorin, suramin, relaxin, gamma interferon (IFN-γ) and -α, relaxin, and decorin reduce muscle fibrosis and, consequently, improve muscle healing, leading to nearly complete recovery of injured muscle (Chan et al., 2005; Chan et al., 2003; Cohn et al., 2007; Foster et al., 2003; Fukushima et al., 2001; Habashi et al., 2011; Li et al., 2005; Negishi et al., 2005; Sato et al., 2003). However, clinical use is hampered by lack of oral dosing formulations, serious side-effect profiles of some of these anti-fibrotic agents and lack of FDA approval for use in humans.

Research results had linked pathologic fibrosis in various tissues to an end-product of the blood pressure-regulating renin-angiotensin system, angiotensin II. Modulation angiotensin II levels with angiotensin II receptor blockers or angiotensin converting enzyme inhibitors has shown decreased fibrosis and improved function in kidney, liver, lung tissue, and the aortic wall (Habashi et al., 2011; Lim et al., 2001; Otsuka et al., 2004; Paizis et al., 2001; Suga et al., 2002). In diseases such as congestive heart failure, injured cardiac muscle is dysfunctional due to increase amounts of fibrosis. Use of angiotensin converting enzyme (ACE) inhibitors or angiotensin receptor blockers to decrease the levels of angiotensin II in myocardium has demonstrated measurable improvement in heart output (Gremmler et al., 2000; Habashi et al., 2011; Swedberg and Kjekshus, 1988). Interestingly, investigators have observed a relationship between treatment for hypertension by the use of medications containing ACE and skeletal muscle health in the elderly. Patients treated with antihypertensive drugs had the unexpected positive side-effect of decreased rates of muscle wasting and a reduction in the relative amount of adipose tissue within their musculature (Onder et al., 2006). In other studies that utilized ACE inhibitors and studies of persons carrying a deletion of ACE gene, a direct effect of the renin-angiotensin system on the skeletal muscle was demonstrated (Folland et al., 2000; Onder et al., 2006). Muscle hypertrophic growth in response to overloading is believed to be modulated by the renin-angiotensin system (Folland et al., 2000; Onder et al., 2006). Other evidence has elucidated the mechanism by which angiotensin II receptor blockade modulates TGF- β1 and showed that it is

implicated in the prevention of muscle regeneration in murine models of Marfan syndrome (Cohn et al., 2007).

Angiotensin II receptor blocker, Losartan potassium (Merck) is FDA approved drug that has minimal side effects, and has been in clinical use for over 20 years (Burks et al., 2011; Fakhfakh et al., 2011). Losartan use has been associated with reduction in fibrosis in several tissues (Burks et al., 2011; Lim et al., 2001; Otsuka et al., 2004; Paizis et al., 2001). Burks et al. have found that blocking angiotensin II type I receptor by Losartan improved muscle remodeling in an aging mouse model, protected against sarcopenia by regulating TGFβ-1 signaling pathways and helped against muscle loss in immobilized hindlimbs by regulating the activity of the insulin-like growth factor 1 (IGF-1)/Akt/mammalian target of rapamycin (mTOR) (Burks et al., 2011). Our group had investigated the effect of Losartan on improving muscle healing after injury in a murine model and have found that Losartan-treated mice exhibited a histological, dose-dependent improvement in muscle regeneration (Bedair et al., 2008). Furthermore, gastrocnemius muscles in mice given the Losartan solubilized in their drinking water and sacrificed 3 or 5 weeks after injury showed a significant reduction in fibrous tissue formation within the area of injury compared to control animals (Bedair et al., 2008). We are performing additional studies to understand the mechanism of action of Losartan as well as potential effects of dosing and timing of application to improve muscle healing in animal models (Kobayashi et al., 2011; Terada et al., 2012).

The clinical implications for this application of angiotensin receptor blockers are potentially far-reaching and include not only sports and military-related injuries, but also diseases like the muscular dystrophies. However, thus far there are no clinical on the use of this drug to optimize healing after muscle injury. We hypothesized that treatment of patients with doses of losartan that does not affect their blood pressure would antagonize the effects of TGF-β1 as we have shown in murine muscles (Bedair et al., 2008) and thus result in a clinically significant reduction of fibrosis formation after a common muscle injury. We have conducted a limited clinical case study on two college athletes to document the effect of losartan on muscle fibrosis and followed their tolerability and time needed to reach strength and flexibility levels that would correlate to

ability to return to play. Furthermore, we followed up on any future recurrences of injury. Below we summarize the preliminary results obtained in this case study.

Case studies

At the initial evaluation in the clinic, a history and physical examination were performed and each of the two patient-athlete. The two patients showed normal neurovascular exam data. They underwent testing that included measurements of isometric hamstring muscle force and flexibility, plain radiographs of the pelvis, to rule out fracture of the ischial tuberosity and magnetic resonance imaging (MRI) (1.5T; GE-Sigma, Waukesha, WI, USA) of the involved muscles was performed to better characterize the injury. After obtaining the patients' consent for treatment, they were started on a 30-day course of losartan (Cozaar®, Merck, Whitehouse Station, NJ) at the oral daily dose of 50mg. In addition to the medication, the subjects underwent routine standard of care rehabilitation supervised by a physical therapist. Return to sports activities progressed from jogging to running and sprinting. The patients were evaluated every 7 days by a study physician (YC) that included measurement of blood pressure and hamstring flexibility and strength as described above. At the conclusion of treatment both patients underwent testing on an isokinetic dynamometer (Biodex II; Shirley, New York) at 60° and 180° per second to assess the torque generating capacity of the hamstring and quadriceps muscles. The MRIs confirmed in both a partial thickness tear of the biceps femoris with surrounding edema. No fracture or significant hematoma was present.

Serial isometric strength and flexibility measurements, performed at different time points, are summarized in **Table 1**. In addition to the isometric tests and isokinetic tests were also

TABLE 1. Results of Strength and Flexibility Measurements

	10 days post injury		3 weeks post injury		4 weeks post injury		7 weeks post injury		9 weeks post injury	
	Strength (%)	Pop angle (°)	Strength	Pop angle	Strength	Pop angle	Strength	Pop angle	Strength	Pop angle
Subject 1	47.2 / 54.1	30 (5)	71.0 / 79.6	5 (5)	85.5 / 92.6	5 (5)	91.5 / 83.5	5 (5)	83.3 / 107.1	5 (5)
Subject 2	59.6 / 60.1	13 (10)	101.4 / 85.5	24 (15)	103.8 / 92.5	15 (12)	110.5 / 99.7	26 (18)	132.5 / 110.8	14 (12)

performed. Both patients reported no side effects while they were taking the losartan and remained normotensive throughout the 30 day course of the medication.

In both patients we initiated treatment with losartan after the acute period of necrosis/degeneration and inflammation (10 days after injury for the first patient and 5 days after injury for the second patient). Patient #2 has demonstrated normal hamstring flexibility and strength by 3 weeks after injury and was without activity-limiting pain at that time (**Figure 4**). Patient #1 was back to normal strength after 9 weeks. While meeting this criteria would normally correlate with appropriate time to return to athletics, both athletes suffered injury at the end of their season and thus a return to sport was not a primary end point. After one year, neither individual has suffered any recurrence of injury.

Extrapolating the time to return to play for hamstring injuries from the previous studies is a difficult and an inexact task as evidenced by the many variables involved and a wide range of time reported in the studies ranging from mean of 14 days to 62 weeks (Askling et al., 2007; 2008; Brooks et al., 2006; Connell et al., 2004; Pomeranz and Heidt, 1993; Slavotinek et al., 2002). For our patients, both have been able to return to normal levels of activity after 9 weeks or even shorter for patient #2. Despite the difficulty of extrapolating from this limited data, in our clinical experience this is a notably shorter period of recovery than expected for patients with a similar degree of recurrent injury.

We are encouraged by the results of this intervention and we believe that Angiotensin receptor blockers may provide clinically effective a non-invasive treatment and safe treatment for improving healing following skeletal muscle injury and it can probably be combined with growth factors and stem cell therapies. However we do recognize the limitations of making treatment decisions based on case reports. We believe that translational clinical research, in the form of a prospective, blinded, placebo controlled randomized clinical trial is necessary to determine the benefits of losartan use in comparison to conventional approaches, to the treatment of hamstring muscle injuries alone. Future research should consider the effects of the losartan on recovery of hamstring flexibility and strength, degree of fibrosis, time to return to full participation in

sports and the frequency of recurrence as well as any adverse effects encountered by otherwise healthy individuals.

Conclusions

Review of recent literature indicates that muscle injury includes well-coordinated and interdependent phases including degeneration, inflammation, regeneration, and fibrosis. Stem cells isolated from different tissues have great therapeutic potential especially when combined with growth factors to modulate their growth and differentiation into certain lineages. From the skeletal muscle, our group has isolated a population termed muscle derived stem cells that has been effective in skeletal muscle repair and has great potential for future therapies for musculoskeletal diseases.

Biologically active signals produced by donor stem cells are believed to elicit response from donor cells and chemoattract host cells to the at the injury site. It is still unclear, though, which host cells are involved in the repair processes after stem cell transplantation. Blood vessel cells, immune and inflammatory cells and resident cells at the injury site all appear to play a role in the regeneration process.

Recent investigations had shown that few donor stem cells are found within the regenerated tissues but this should not detract from the potential of stem cells. Current findings indicate that the improvement in function is probably caused by donor stem cell paracrine signaling. (Chen et al., 2008; Gharaibeh et al., 2011; Santhanam et al., 2007; Togel et al., 2007; Yoon et al., 2005).

It is clear that the multipotentiality of the stem cells may not represent a major determinant for the success of stem cell therapy. This idea challenges current dogma that suggests that embryonic stem cells may have an advantage over adult derived stem cells because of their higher level of multipotentiality. We speculate that it is the stem cells' resistance to oxidative stress and paracrine signaling capacity that lead to their increased ability to attract host cells and it is this feature that is key to successful stem cell therapy.

Choosing the most robust stem cell population with the greatest potential for differentiation toward other cell lineages will continue to be of valuable interest to restore the structure and function of damaged tissues. Furthermore, additional efforts to isolate factors involved in the paracrine action of different stem cells population under different conditions will very likely be utilized by future therapeutic applications.

TGF-β1 is a cytokine that plays a significant role in the formation of fibrotic tissue in skeletal muscle and other tissues. Antifibrotic agents including angiotensin receptor II blocker (such as losartan) are very effective in reducing muscle fibrosis and are likely to be a strategy that can be used in clinical therapies in the future. We believe that more rigorous clinical trial using losartan is necessary to determine the benefits of losartan use in comparison to conventional approaches alone for the treatment of skeletal muscle injuries. This research should also consider the effects of the losartan on recovery of muscle flexibility and strength, amount of fibrosis as well as any adverse effects encountered by otherwise healthy individuals. Furthermore, future experiments on the use of losartan should compare the effects of using losartan on muscle healing when used with or without anti-inflammatory drugs that seem to delay muscle regeneration.

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Figures 1-4

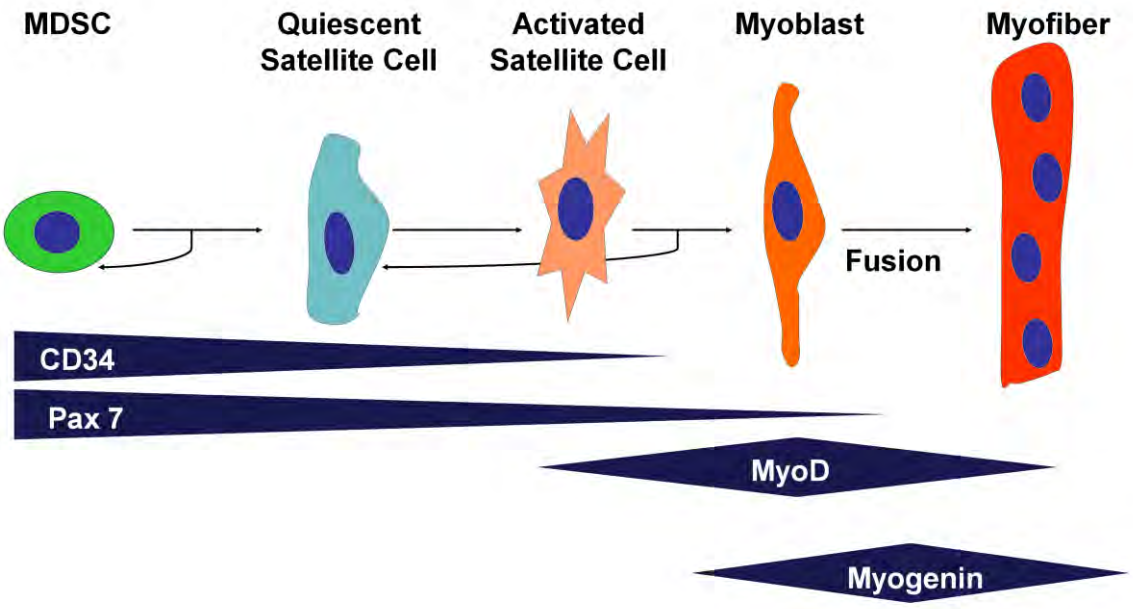

Figure 1. Generalized scheme of myogenic differentiation. Other markers are used by different investigators. (Figure adapted from Deasy, Jankowski and Huard. 2001. Blood Cells, Molecules and Diseases. 27(5): 924-933).

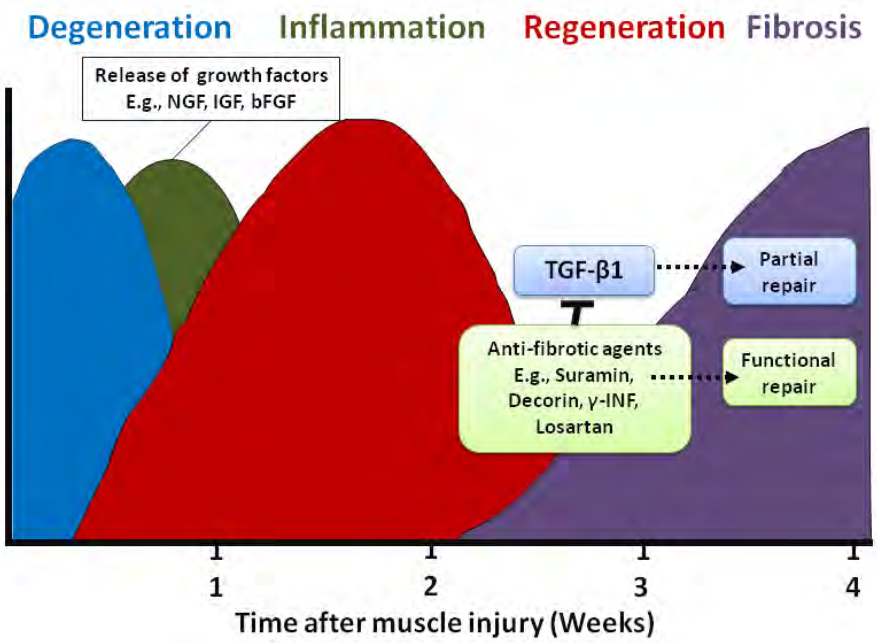

Figure 2. Healing process in the skeletal muscle. Several overlapping phases are accompanied by the release of growth factors that modulate regeneration and formation of fibrotic tissue. Use of anti-fibrotic agents minimizes muscle scarring and leads to better functional outcome.

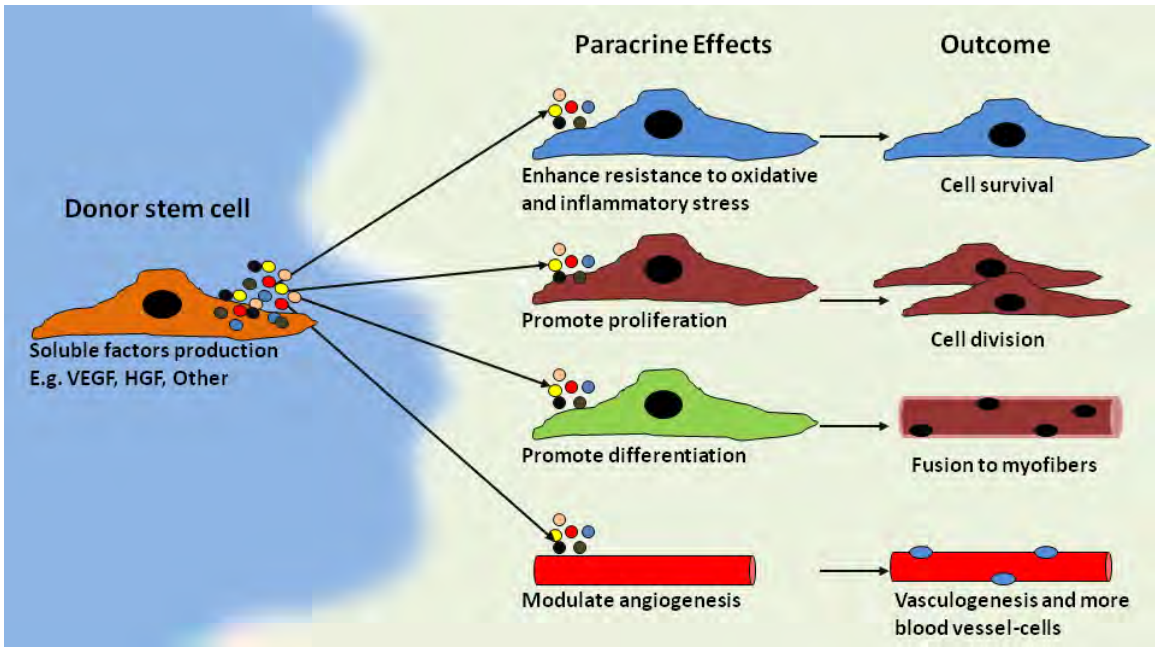

Figure 3. Schematic of potential scenario of events taking place during cell therapy. Donor stem cells proliferate, differentiate, apoptose, senesce or more importantly produce trophic factors that would have autocrine and paracrine effects on other donor and host cells. Furthermore, certain factors (e.g. VEGF) promote agniogenesis which can positively affect other processes.

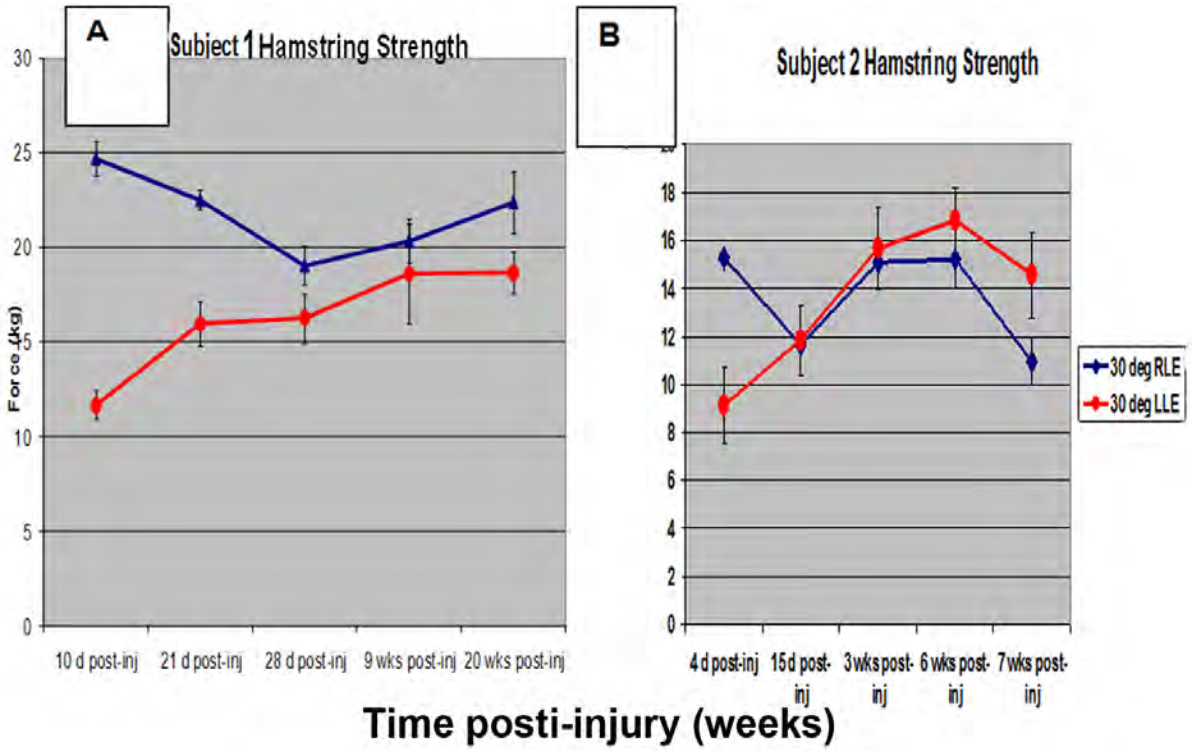

Figure 4. Hamstring strength in two hamstring injury patients treated with Losartan. (Affected leg readings are charted in Red).

TABLE 1. Results of Strength and Flexibility Measurements										
	10 days post injury		3 weeks post injury		4 weeks post injury		7 weeks post injury		9 weeks post injury	
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